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14. ABSTRACT
This study was designed to identify human mutation that caused Exertional and/or environmental heat stroke (EHS) and exertional rhabdomyolysis (ER) and to develop new interventions to prevent EHS and ER. We identified RyR1 mutations as a major cause of exertional rhabdomyolysis. 82% of the individuals that were enrolled in the study that had a family history of MH were positive for disease-associated mutations in the RYR1 gene, while 10.5% of index cases enrolled for a history of unexplained or recurrent ER were positive for disease-associated mutations in the RYR1 gene. All of these cases were CHCT positive. Although AICAR was not effective in preventing isoflurane or heat induced episodes in hyper-metabolic response in pigs homozygous for an RyR1 mutation, AICAR was effective in preventing heat-induced hyper-metabolic responses in the heterozygous mouse model of malignant hyperthermia.

15. SUBJECT TERMS
Exertional Heat Stroke, Exertional Rhabdomyolysis, Malignant Hyperthermia , genetic analysis of type 1 Ryanodine Receptor (RYR1) and 5-Aminoimidazole-4-carboxamide ribotide (AICAR).

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Table of Contents

Page

1. INTRODUCTION: Exertional and/or environmental heat stroke (EHS) and exertional rhabdomyolysis (ER) have been reported in patients with a diagnosis of Malignant Hyperthermia (MH) susceptibility. MH is a rare, inherited subclinical myopathy identified by an unexpected hypermetabolic reaction during anesthesia. The gene most closely linked to MH codes for the ryanodine receptor type 1 (RYR1) in skeletal muscle. It has also been associated with other candidate genes including the voltage dependent L-type alpha-1S subunit calcium channel (CACNA1S) and calsequestrin 1 (CASQ1). The RYR1 gene encodes the skeletal muscle calcium (Ca^{2+}) release channel. Mutations in this gene have been associated with different human muscle disorders, ranging from asymptomatic MH to highly penetrant core myopathy [1, 2]. It is thought that mutations in the RYR1 gene are associated with 50-80% of cases of MH susceptibility (MHS), depending on the study population and method of screening[1]. The frequency of RYR1 gene mutations in ER and EHS is unknown.

In a transgenic mouse model for MH, AICAR was found to be effective in treating a heat-induced MH crisis. In this study, AICAR was tested in an *in vivo* MHS swine model, as well as tested *in vitro* on isolated MHS swine muscle preparations. In the human study, our goal was to identify mutations associated with enhanced susceptibility to EHS/ER/MH by enrolling subjects diagnosed with these conditions and performing genetic screening in proteins that play important roles in skeletal muscle Ca^{2+} regulation.

2. KEYWORDS: Heat stroke, exertional rhabdomyolysis, malignant hyperthermia, interventions, ryanodine receptor, voltage dependent calcium channel, AICAR, dantrolene

3. OVERALL PROJECT SUMMARY: Specific Aim 1. Identify human RyR1 mutations associated with enhanced susceptibility to ER/EHS.

Task 1: Screen for RyR1 mutations

Milestone 1: Show that RyR1 mutations underlie cases of enhanced susceptibility to heat stroke. In total, 45 subjects where enrolled in this study to identify correlations between EHS/ER/MH and genetic variants in the proteins that play important roles in skeletal muscle calcium (Ca^{2+}) regulation.(Appendix 1) Of these volunteers, 32 were index cases and 13 individuals were family members of index cases. Three publications have resulted from this study. (Appendix).

Table 1. Summary of Genetic Variants Identified (n=45)

Gene Screened	Total Subjects Screened	Total Variants Identified	Number of Subjects with Variants	Subjects with Multiple Variants	Index Cases with Variants	Family Members with Variants
RYR1	45	30	25	4	18	8
CACNA1S*	21	8	7	1	7	0
CASQ1*	21	2	2	0	2	0

*all variants identified in CACNA1S and CASQ1 were variants reported in general population.

RYR1 gene mutations and variants have been found in 9 index cases, which account for approximately 30% of all enrolled individuals, not including family members. Such a low yield is likely due to the clinical heterogeneity of our study subjects, which include subjects with a history of ER and EHS, as well as MH susceptibility. The frequency of RYR1 mutations is notably different between subjects with different phenotypes. In table 2, subjects are classified into several sub-types depending on clinical data and diagnostic phenotypes.

Table 2. Phenotype and Genotype Correlation

Clinical data and Phenotype	MHS associated RYR1 mutation	RYR1 variants of unknown significance	New RYR1 variants	Negative for all screened genes
Personal or family history of MHS, CHCT(+)	5	0	0	0
Personal or family history of MHS, no CHCT*	3 6 fam. memb.	0	0	3
Personal history of ER or HS, CHCT (+)	1	1	2	5
Personal history of ER or HS, CHCT (-)	0	1	2	7

Personal history of ER or HS, no CHCT*	0 2 fam. memb.	0	0	2

*Included family members are identified separately from index cases.

All subjects (N=5) who had personal and or family history of MH followed by positive CHCT results carried disease associated mutations in the RYR1 gene. Disease associated mutations were also found in 50% of subjects (N=6) with compelling clinical history of MHS, but no CHCT results. Overall, 82% (9 of 11) of MHS subjects were positive for disease associated RYR1 mutations. Although this is a small sample size, data suggest that both clinical history and positive CHCT results are important predictors of MH, and that these subjects are likely to have disease associated mutations in the RYR1 gene.

Our data further revealed that the presence of the second rare variant in the RYR1 may serve as a modifying factor. One patient, who died of an awake MH-like episode but was known to be MH susceptible, had the Phe41Ser and the Gly2434Arg known MH-causative mutation. Of these two variants, the Gly2434Arg is one of the most common pathogenic mutations in the RYR1 gene. Interestingly, Phe41Ser has recently been reported in a family with congenital myopathy.[4] These data are in agreement with our previous studies[5] and suggest that the presence of the second variant may have contributed to the fatal outcome in this case.

The majority of enrolled cases (N=21) were referred due to repeated ER and or EHS. Of those ER cases, 47% (9 of 19 subjects) tested positive and 53% (10 of 19 subjects) were negative for CHCT. Disease associated RYR1 gene mutations, Arg2454Cys and Val4842Met, were found in two independent families with ER and positive CHCT. The Arg2454Cys is a recurrent, pathogenic mutation associated with MHS in independent studies[2], whereas the Val4842Met variant was previously reported in a family with core myopathy.[6]

Two new variants, Ala2533Thr and Tyr4850Stop, were identified in ER/CHCT positive subjects. To determine if these new variants contribute to the ER/MHS phenotype, we used SIFT and PPhen programs to predict its functional effect. The Ala2533Thr variant predicted to be deleterious by SIFT, whereas PPhen analysis predicted the variant to be benign. The Tyr4850Stop is a frameshift mutation that is likely to lead to truncated RYR1 protein. Such a deleterious change will result in an almost 50% reduction of the functional protein. However, most if not all MH-associated mutations are missense mutations that cause gain of function, and it is unlikely that the Tyr4850Stop change will lead in enhanced calcium release. Thus, the contributions of the two new variants remains to be determined.

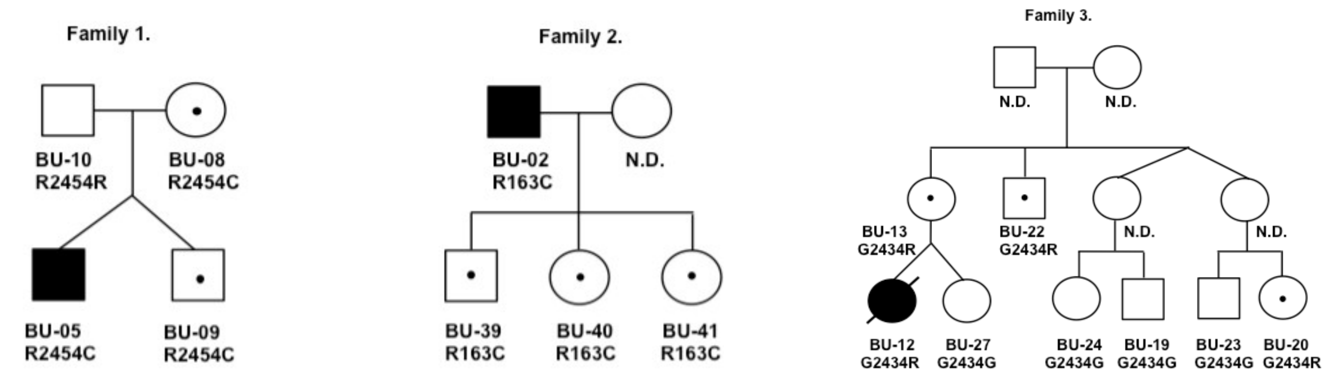
One rare (Thr4823Met) and two new variants (Thr4259Ala, and Glu4410Asp) were identified in subjects with a history of ER and a negative CHCT result. While highly sensitive (over 90%), the contracture tests have only 78% specificity.[3] Given this diagnostic limitation of the CHCT, it is possible that subjects carrying these variants may be at risk for MH despite the CHCT negative result. On the other hand, these changes might be rare benign variants that are not associated with ER and/or MH. Functional studies to characterize the pathogenic consequences of these mutations, and to analyze phenotype-genotype relationship are important to determine whether the newly identified variants contribute to subjects' clinical presentation of ER and/or MHS.

Overall, about 10.5% of the subjects with the ER/EHS diagnosis and a positive CHCT had disease associated mutations in the RYR1 gene, indicating the importance of this test for the differential diagnosis of myopathies. The screening of ER subjects for other MH-associated genes was negative, which suggests that the contribution of mutations in CACNA1S and CASQ1 in ER and EHS is not significant. Therefore, other genetic factors are likely to contribute to ER and EHS.

Of the 20 subjects who did not have a muscle biopsy and CHCT performed for clinical diagnostic purposes, 13 were family members of index cases who had previously had a muscle biopsy and positive CHCT.

Of the family members enrolled, 8 had familial mutations in the RYR1 gene. (Figure 1).

Figure 1 – Pedigrees of three unrelated MHS and ER families.



Filled symbols denote index cases.
Filled symbols with a bisecting line indicates the individual died of an MH episode
Symbols with single dots indicate subjects with RYR1 variants.
R – Arginine, C- Cysteine, G- Glycine.
Numbers correspond to amino acid positions of RYR1 protein.

All identified variants in the CACNA1S and CASQ1 genes were reported in NCBI single nucleotide polymorphisms (SNP) database[7] and in genome database that cover genome of several thousands of healthy individuals.[8] The CACNA1S variant (Val1449Gly) that was previously identified as a novel variant, is now being reported as a variant in the general population.

To date, RYR1 and CACNA1S gene variants were found in several ER subjects. Table 1 provides a summary of identified variants. Table 2 summarizes the correlation between identified RYR1 variants and clinical phenotype. A full table of the summarized data can be found in Appendix 1

Milestone 2 Publish findings

Specific Aim 2: Develop interventions to prevent heat Induced sudden death associated with RyR1 mutations.

AICAR (as discussed below was not efficacious in the MHS swine model. We , therefore examined the effects of agents that did not affect AMPK activity directly but had similar effects to AICAR on RYR1 activity. We found that low dose rapamycin and synthetic ligand for rapamycin (SLF) could prevent the heat induced MH response in YS mice (**Fig. 2**) and improved muscle function in WT mice (Lee et al, 2014, attached.).

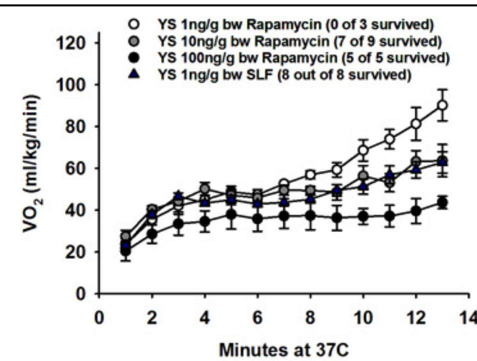


Fig. 2. Rapamcyin and SLF prevent heat- induced hypermetabolic responses in the YS mouse model.

Milestone 6 Publication of Milestone 5

Task 4. Assess the ability of AICAR to prevent MH response in MHS susceptible pigs. AICAR did not rescue the MH pigs.

Milestone 7: Establish ability of AICAR to rescue MHS pigs.

While AICAR was found to be an effective drug for preventing and rescuing a heat-induced MH crisis in a transgenic mouse model, these findings could not be duplicated in the *in vivo* swine model of MH. AICAR was shown to have no efficacy for either pre-treatment or rescue of anesthesia- or heat-induced MH in the swine model. The results from the *in vitro* studies from MHS swine muscle indicate that AICAR had no effect at both low and high concentrations on muscle tension development in basal conditions, nor did AICAR influence responses induced by caffeine and halothane challenges. This is consistent with the previous report that AICAR is ineffective when administered intravenously in the MHS pig model. The reason for this may be that the pigs are homozygous for the MH mutation. This suggests the need to test this in heterozygous pigs but these studies were not budgeted.

4. KEY RESEARCH ACCOMPLISHMENTS:

HUMAN STUDY

- 82% of index cases enrolled for a personal or family history of MH were positive for disease-associated mutations in the RYR1 gene.

10.5% of index cases enrolled for a history of unexplained or recurrent ER were positive for disease-associated mutations in the RYR1 gene. All of these cases were CHCT positive.

- Manuscript in print in *Anesthesia and Analgesia*. "Death in the Emergency Department: An unrecognized awake MH-like reaction in a six year old". (Appendix 2)
- Manuscript in print in *Molecular Genetics and Genomic Medicine*. "Exome analysis identifies Brody myopathy in a family diagnosed with malignant hyperthermia susceptibility." (Appendix 3)

SWINE STUDY

- AICAR is ineffective at preventing or rescuing a heat- or anesthesia-induced MH crisis in the *in vivo* swine model.
- AICAR at 1mM and 10mM did not alter basal tension of *in vitro* MHS swine muscle strips when compared to baseline tension of untreated control strips.
- AICAR (1 and 10mM) did not significantly alter halothane- or caffeine-induced contractures in MHS swine muscle *in vitro*.

MOUSE STUDY:

- AICAR prevents heat induced deaths in YS mice, but appears to be less efficacious for preventing the isoflurane induced response in either the mice or the pigs.
- The AICAR prevention of heat induced death in the YS mouse model could be mimicked with low dose rapamycin and SLF, drugs that do not directly alter AMPK activity.

5. CONCLUSIONS:

HUMAN STUDY

Of the 32 unrelated individuals enrolled in the study, 11 were enrolled for a history of MH (personal or family), and 21 were enrolled for a history of ER or EHS. 82% of those with a history of MH were found to have disease associated mutations in the RYR1, whereas only 10.5% of those with a history of ER or EHS were found to have disease associated mutations in the RYR1. Additionally, disease associated mutations were detected only in ER/EHS subjects with a positive CHCT. This highlights the importance of the CHCT as a diagnostic tool for myopathies related to calcium dysregulation. This also indicates that ER and EHS are genetically heterogenous disorders that require further studies to identify other candidate genes.

MOUSE STUDY: AICAR, low dose rapamycin and SLF improve muscle performance and prevent heat induced deaths in a mouse model of MH. They are less effective in preventing anesthetic induced responses in these mice.

SWINE STUDY

AICAR, when administered as a pretreatment or as a rescue drug intravenously, was not effective as a treatment for heat-induced MH in the MH susceptible swine. This finding appeared to be in conflict with data obtained in the MH susceptible transgenic YS mouse model. We hypothesized that in the pig model AICAR (600mg/kg IV) may not achieve an effective concentration in the swine skeletal muscle. To test this possibility, we examined the effect of AICAR on isolated muscle preparations from the MHS pigs and found that the drug had no effect on muscle tensiondevelopment either in basal conditions or when agonists were applied.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

1. Lavezzi WA, Capacchione JF, Muldoon SM, Sambuughin N, Bina S, Steele D, Brandom BW. Case report: death in the emergency department: an unrecognized awake malignant hyperthermia-like reaction in a six-year-old. *Anesth Analg*. 2013; 116: 420-423.

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3. Potts L, Longwell J, Bedocs P, Sambuughin N, Bina S, Cooper P, Carroll C, O'Connor F, Deuster P, Muldoon S, Hamilton S, Capacchione J. Improving Awareness of Nonanestheia-Related Malignant Hyperthermia Presentations: A Tale of 2 Brothers. *Anesth Analg Case Reports*. 2014; 3(2):23-26.

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Synthesis to Improve Skeletal Muscle Function. *J. Biol. Chem.* published 22 July 2014, 10.1074/jbc.M114.586289

7. INVENTIONS, PATENTS AND LICENSES: List all patents and licenses applied for and/or issued. Each entry must include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

8. REPORTABLE OUTCOMES: see publication in section 6.

9. OTHER ACHIEVEMENTS:

"Nothing to report."

10. REFERENCES

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8. Muldoon, S., et al., *Exertional heat illness, exertional rhabdomyolysis, and malignant hyperthermia: is there a link?* Curr Sports Med Rep, 2008. **7**(2): p. 74-80.

11. APPENDICES:

Genetic Screening Results

Report ID	Clinical history	CHCT Results	Index or Family members	Results of genetic screening*		
				RYR1	**CACNA1S**	**CASQ1**
BU-01	Exercise induced rhabdomyolysis, heat related death in a family member	Positive	index	**Gly4820Arg**	N.A.	N.A.
BU-02	Muscle pain with exercise post MH event	Positive	index	**Arg163Cys**	N.A.	N.A.
BU-03	Exercise induced rhabdomyolysis, MHS	Positive	index	Negative	Negative	Negative
BU-04	Exercise and heat intolerance, muscle cramping and MH	Positive	index	**Asp3986Glu**	N.A.	N.A.
BU-05	Repeated Exercise induced rhabdomyolysis, history of MHS	Positive	index	**Arg2454Cys**	N.A.	N.A.
BU-06	Repeated Exercise induced rhabdomyolysis, family history of MH	N.D.	index	**Gly2434Arg**	N.A.	N.A.
BU-07	Death due to MH-like event	N.D.	index	Negative	N.A.	N.A.
BU-08	No known clinical history	Positive	Mother of BU-05	**Arg2454Cys**	N.A.	N.A.
BU-09	Cardiac arrest during surgery	N.D.	Twin brother of BU-05	**Arg2454Cys**	N.A.	N.A.

BU-10	No known clinical history	N.D.	Father of BU-05	Negative for Arg2454Cys familial mutation	N.A.	N.A.
BU-11	Traumatic MH Episode	N.D.	index	**Arg2163His**	N.A.	N.A.
BU-12	Death due to MH and Exercise/heat intolerance	N.D.	index	Phe41Ser **& Gly2434Arg**	N.A.	N.A.
BU-13	MH and heat related death in family member	N.D.	Mother of BU-12	**Gly2434Arg**	N.A.	N.A.
BU-14	Exercise and heat intolerance, MHS	Positive	index	Tyr4850Stop	Arg1658His	Negative
BU-15	Repeated Exertional rhabdomyolysis	Positive	index	**Val4842Met**	N.A.	N.A.
BU-16	Exercise and heat intolerance	Negative	index	Negative	Leu458His Val1449Gly	Negative
BU-17	Repeated Exertional rhabdomyolysis	N.D.	index	Negative	Negative	Negative
BU-18	Exercise intolerance, heat stroke and mother had suspected MH episode	N.D.	index	Negative	Negative	Negative
BU-19	MH and heat related death in family member	N.D..	1st Cousin of BU-12	Negative for Gly2434Arg familial mutation	N.A.	N.A.
BU-20	MH and heat related death in family member	N.D.	1st cousin of BU-12	Negative for familial mutation, Gly2434Arg	N.A.	N.A
BU-21	Repeated Exertional rhabdomyolysis	Positive	index	Negative	Negative	Negative
BU-22	MH and heat related death in family member	N.D.	Uncle of BU-12	**Gly2434Arg**	N.A.	N.A.
BU-23	MH and heat related death in family member	N.D.	1st Cousin of BU-12	Negative for Gly2434Arg familial mutation	N.A.	N.A.
BU-24	MH and heat related death in family member	N.D.	1st Cousin of BU-12	Negative for Gly2434Arg familial mutation	N.A.	N.A.
BU-25	Heat and Exercise intolerance	N.D.	index	Negative	Negative	Negative
BU-26	Exertional Rhabdomyolysis/ Heat & Exercise Intolerance	Negative	index	Thr4259Ala	Negative	Negative
BU-27	MH and heat related death in family member	N.D.	Twin sister to BU-12	Negative for Gly2434Arg familial mutation	N.A.	N.A.
BU-28	Repeated Exertional rhabdomyolysis	Positive	index	Negative	Leu1800Ser	Negative
BU-29	Repeated Exertional rhabdomyolysis	Positive	index	Ala933Thr Ser1342Gly Ala2533Thr	Negative	Negative
BU-30	Repeated Exertional rhabdomyolysis	Positive	index	Negative	Leu458His	Met87Thr
BU-31	Repeated Exertional rhabdomyolysis	Positive	index	Negative	Negative	Negative
BU-32	Repeated Exertional rhabdomyolysis	Negative	index	Arg1109Lys Thr2787Ser	Negative	Negative
BU-33	Repeated Exertional rhabdomyolysis	Negative	index	Negative	Negative	Negative
BU-34	Repeated Exertional rhabdomyolysis	Negative	index	Thr4823Met	Arg1658His	Negative
BU-35	Repeated Exertional rhabdomyolysis	Negative	index	Negative	Arg1539Cys	Negative
BU-36	Repeated Exertional rhabdomyolysis	Negative	index	Glu4410Asp	Arg1658His	Negative
BU-37	Repeated Exertional rhabdomyolysis	Negative	index	Met923Val & Pro4501Leu	Negative	Negative

BU-38	MHS, exercise intolerance	Positive	index	**Gly2434Arg**	N.A.	N.A.
BU-39	Muscle pain with exercise post-MH event; MHS family member	N.D.	Child of BU-02	**Arg163Cys**	N.A.	N.A.
BU-40	Muscle pain with exercise post-MH event; MHS family member	N.D.	Child of BU-02	**Arg163Cys**	N.A.	N.A.
BU-41	Muscle pain with exercise post-MH event; MHS family member	N.D.	Child of BU-02	**Arg163Cys**	N.A.	N.A.
BU-42	Suspicious MH-like clinical episode, heat intolerance, cramping	N.D.	index	Gln3756Glu	Negative	Tyr140Phe
BU-43	Suspicious MH-like clinical episode	Positive	index	**Ala2350Thr**	N.A.	N.A.
BU-44	Repeated Exertional rhabdomyolysis	Negative	index	Negative	Negative	Negative
BU-45	Repeated Exertional rhabdomyolysis	Negative	index	Negative	Negative	Negative

* - MHS causative mutations are in bold; newly identified amino-acid variants are underlined. N.D. – not determined. N.A. – not applicable

Death in the Emergency Department: An Unrecognized Awake Malignant Hyperthermia-Like Reaction in a Six-Year-Old

Wendy A. Lavezzi, MD,* John F. Capacchione, MD,† Sheila M. Muldoon, MD,† Nyamkhishig Sambuughin, PhD,† Saiid Bina, PhD,† Deanna Steele, MS, CGC,‡ and Barbara W. Brandom, MD§||

A healthy 6-year-old boy developed lower extremity rigidity, trismus, and fever after playing in a splash pool. On arrival in the emergency department, he appeared to be seizing. An endotracheal tube was emergently placed using succinylcholine. Cardiac arrest followed. He could not be resuscitated. Postmortem genetic analysis found a novel *RYR1* variant. Family testing revealed the same variant in his father who also had muscle contracture testing diagnostic for susceptibility to malignant hyperthermia and central core disease diagnosed histologically. Because there was no exposure to volatile anesthetics before the onset of symptoms, this is a case of "awake" malignant hyperthermia worsened by succinylcholine. (Anesth Analg 2013;116:420–23)

Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle characterized by a hypermetabolic response to inhaled anesthetics and succinylcholine resulting from increased myoplasmic calcium.[1] To some, the concept of MH-like episodes without anesthetic exposure is controversial, despite literature to suggest otherwise.[2–4] We describe a case of apparent awake MH that, after succinylcholine administration during resuscitation, led to cardiac arrest and death. Postmortem genetic analysis revealed the presence of a novel variant in the ryanodine receptor type 1 (*RYR1*) gene, the gene most strongly associated with MH susceptibility.[5] This case implies that other health care providers, especially first responders who use succinylcholine, should be more conscious of the risk of succinylcholine administration in patients, particularly children, presenting with muscle rigidity and hyperthermia without exposure to anesthetic drugs.

CASE DESCRIPTION

A 6-year-old Caucasian boy weighing 20 kg, who was not febrile or acutely ill, was playing outside on a hot, humid day in a splash pool for less than 10 minutes when he suddenly developed an inability to bend his legs. He told his mother that his heart felt like it was pounding out of his chest. His mother measured his temperature at 104°F and tried, unsuccessfully, to cool him with a cold shower, ice packs, and cold oral fluids. She therefore drove the child to the local emergency department (ED). En route, the child, although awake, complained of inability to speak clearly because he was unable to open his mouth. On admission to the ED, his rectal temperature exceeded 108.9°F, and he was sweating profusely. His respiratory rate was 60 breaths per minute, oxygen saturation 99%, arterial blood pressure 99/52 mm Hg, and heart rate 190 beats per minute. He appeared to have generalized seizures.

Benzodiazepine (1 dose of diazepam 5 mg; lorazepam 1 mg 4 times separated by 1 minute each for a 4-mg total dose) and other sedative hypnotics (etomidate 3 mg 2 times separated by 1 minute for a 6-mg total dose; propofol 10 mg 2 times separated by 3 minutes for a 20-mg total dose) were given IV over an 18-minute period without an effect on the presumed seizure activity. At 6 minutes into the 18-minute treatment interval, atropine (0.4 mg IV) was also given, presumably to decrease the oral secretions, which were obvious on admission to the ED. Cooling measures with IV fluids and external ice packs were attempted, but could not reduce the fever. Respiration was assisted and tachycardia continued throughout this period.

Because the child was in respiratory distress, succinylcholine (20 mg IV) was administered to place an endotracheal tube 19 minutes into the treatment. Jaw relaxation did not occur as anticipated, and another dose of succinylcholine (20 mg IV) was given 1 minute later. An acetaminophen suppository (360 mg) was also given at the same time as the second dose of succinylcholine. Bradycardia occurred almost immediately, then asystole. Advanced cardiac life support was instituted (25 minutes after the onset of treatment with diazepam) with atropine (0.6 mg IV) given 5 minutes after the second dose of succinylcholine. A ceftriaxone (1 g IV) infusion was started at the same time. A second dose of atropine (0.6 mg IV) was given 6 minutes into the resuscitation (beginning of advanced cardiac life support). Bicarbonate (20 mEq IV) was given at 4, 8, and 17 minutes into the resuscitation, and another 40 mEq was given

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3 minutes later. Epinephrine (0.2 mg IV) was administered at 14, 15, and 21 minutes into the resuscitation. His laboratory values were significant for plasma potassium 9.4 mEq/L (10 minutes after the second dose of succinylcholine, and 5 minutes into the resuscitation), total creatine kinase 981 U/L with 19.1 U/L MB creatine kinase, and white blood cell count of $10.4 \times 10^3/\mu L$. (Other laboratory values 5 minutes into resuscitation were sodium 140, chloride 101, glucose 163, calcium 8.0, magnesium 2.5, blood urea nitrogen 20, creatinine 1.0.) Despite the multiple doses of atropine, epinephrine, and bicarbonate, a spontaneous cardiac rhythm never returned, and the arterial blood gases at 32 minutes into the resuscitation were pH 7.155, P_{CO_2} 166, and P_{O_2} 15.9. The rectal temperature was still 108°F.

Death was pronounced 1 hour after arrival in the ED, and 2 hours after his first presenting symptoms. An autopsy was performed within 24 hours. There were no gross pathologic findings or obvious signs of infection. Blood and tissue cultures were negative for significant organisms, microscopic studies were unremarkable, and toxicologic studies were negative for alcohol and drugs, other than what was administered in the ED. Vitreous electrolytes showed mild dehydration.

A retrospective review of the pediatrician's records of the decedent revealed that he did not walk until 17 months of age, and that he had prominent lumbar lordosis; however, no muscle disorder was ever diagnosed. A genetic consultation at 3 years of age found no evidence for spinal muscular atrophy. Approximately 2 to 3 weeks before his death, the decedent experienced "overheating" and stomach cramps while playing outdoors in the heat. This resolved with rest and drinking cold water. Otherwise, he was well with no febrile illnesses. He periodically complained of leg pain. Two of his siblings and his father also had lumbar lordosis and did not walk until age 17 months. A 10-month-old female sibling had no lordosis and normal motor milestones. The family denied a history of MH, heat intolerance, or exercise intolerance. However, based on the negative autopsy and postmortem studies, the presence of muscle rigidity with extremely high temperature and adverse response to succinylcholine, the forensic pathologist determined MH to be the cause of death. As a result, the family was referred for further MH-related diagnostic testing.

A postmortem liver specimen was analyzed for *RYR1* mutations. A novel *RYR1* variant, Gly4820Arg, was found in exon 100. The decedent's parents, grandparents, and siblings were screened for the same variant. The same *RYR1* variant was only found in blood samples from the decedent's father and 2 siblings with lumbar lordosis. The decedent's father underwent a muscle biopsy and caffeine-halothane contracture test (CHCT), which was strongly positive for MH: mean contracture response of 3.85 g in the presence of 3% halothane and 1.32 g in the presence of 2 mM caffeine. Histology of the father's muscle sample revealed central core disease (CCD).

DISCUSSION

This child presented to the ED with rapidly increasing core temperature, profuse sweating, increased minute ventilation, tachycardia, trismus, and extremity rigidity. Attempts to break the presumed fever-induced seizure with benzodiazepines, other sedatives, and external cooling proved unsuccessful. Neurologic disease, including seizures, could produce these signs. Severe rigidity can be neurologic or muscular in origin. If seizures are not easily treated, pharmacologic intervention to control the airway may be needed. Airway intervention is also necessary for respiratory distress. This child's signs and symptoms were similar to those of status epilepticus, but they were also very similar to the much less common condition, acute fulminant MH, which is typically associated with the administration of potent volatile inhaled anesthetics and/or succinylcholine.

Because the respiratory rate of 60 breaths per minute and heart rate of 190 beats per minute persisted despite attempts to stop the presumed seizures, the ED personnel determined it necessary to secure the child's airway. The rapidity with which the temperature increased (not suggestive of an infectious etiology), and the history that the child was delayed in reaching the usual motor milestones and potentially myopathic, went unappreciated. Although succinylcholine is an appropriate drug to facilitate the emergent placement of an endotracheal tube, succinylcholine also has a Food and Drug Administration Black Box Warning because of potential hyperkalemic cardiac arrest for children (especially males) younger than 8 years, due to unrecognized myopathies.[6] Given the extreme temperature increase, coupled with the other signs and symptoms, dantrolene therapy as part of the resuscitation would have been appropriate and potentially life-saving. It was the combination of hyperthermia and rigidity that was most concerning for possible MH. Unlike MH, exertional or environmental heat stroke does not typically present with muscle rigidity; however, dantrolene administration in environmental heat stroke cases refractory to conventional cooling therapy may be useful.

The ED physicians taking care of this child were placed in an unenviable situation. They needed to act quickly to secure the airway of a rigid, hyperthermic, apparently seizing child in respiratory distress who was refractory to antiseizure medications. For unknown reasons, the child's history of motor delay was missed. The administration of succinylcholine likely increased rigidity and precipitated hyperkalemic cardiac arrest. It is unlikely that these ED physicians were aware that MH-like reactions could occur in the absence of anesthesia. Confirmation of an *RYR1* variant in the decedent, as well as in the father (de novo mutation) and 2 siblings, who likewise had histories of developmental motor delays, raised the suspicion that this tragic episode was an "awake MH reaction."

Although the *RYR1* variant identified in this case had not been reported or characterized as pathogenic, a different amino acid substitution (Gly4820Trp) at the same locus in a known "hot spot" region (exon 100) of the *RYR1* had been described in association with CCD.[7] The father's CCD histopathology and strongly positive CHCT (North American MH protocol) confirmed the diagnosis.[7,8] CCD is a known MH-associated myopathy. Furthermore, heat and/or exercise stress trigger nonanesthetic-related MH-like reactions in mouse and swine models of MH.[9–11] Finally, it should be noted that the lack of MH family history is not a guarantee for lack of proband MH sensitivity, because de novo mutations, as in this case, do rarely occur.

Given that the *RYR1* variant identified in this case shared the same locus as a previously described mutation in an unrelated proband, and given that the decedent's father, who shared the same variant, also had a strongly positive CHCT and CCD histopathology, the variant should be considered a mutation causal for MH. Although the decedent's father denied a history of complications during general anesthesia or history of muscle weakness or pain, his wife reported that he could not complete any routine gardening or yard work without taking an unusual number of rest periods. It seemed that over the course of his lifetime he had learned to modulate his fatigue during physical activities, which for him, at least, was not unusual. These parents have appropriate concerns for their 2 children who carry the same mutation, and they have been educated regarding MH signs, symptoms, and triggers. All MH-susceptible family members now wear medical alert bracelets, and the children's school nurse has been informed of their MH susceptibility and how their brother died.

The incidence of MH during anesthesia is rare, estimated between 1 in 4200 and 250,000 general inhaled anesthetics.[12] However, the predicted incidence of MH-causative genetic variants in the general population is estimated at 1 in 2000 to 3000.[13,14] This is more than one would expect based on the clinical occurrence of MH during anesthesia.[15] Because of variable expression and penetrance, people who are MH susceptible may undergo anesthesia with MH-triggering drugs but still not experience an MH reaction. In fact, Larach et al.[16] reported that 50.7% of subjects in a cohort of subjects in the North American MH Registry had 2 or more unremarkable anesthetics before experiencing an MH reaction. These data suggest that the risk of MH in the general population is far greater than one would expect based solely on clinical presentation during anesthesia. In fact, similar cases of awake MH in children, with and without recognized myopathies, have been described.[3,4]

Because MH is a genetic disorder of skeletal muscle calcium regulation, it might be naive to assume that potent volatile inhaled anesthetics and succinylcholine are the only drugs that precipitate MH episodes. Succinylcholine should not be used in patients who present to the ED with rigidity and hyperthermia unrelated to anesthesia. It is possible that anesthetic drugs are just one of many environmental factors that can precipitate MH reactions. A broader definition of the MH syndrome to include nonpharmacologic environmental triggers is critical because health care professionals who do not practice anesthesia may be called upon to treat MH-related crises that occur without anesthetic exposure. Medical examiners need to consider MH when dealing with deaths marked by hyperthermia, rhabdomyolysis, and acidosis in young, healthy patients with the suggestion of underlying myopathies. Finally, it is critical for the anesthesia community to provide MH-related educational programs with guidelines to ED physicians and others who administer succinylcholine in emergent situations and to make them aware of the possible dangers of administering succinylcholine to patients who are hyperthermic and rigid.

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ORIGINAL ARTICLE

Exome analysis identifies Brody myopathy in a family diagnosed with malignant hyperthermia susceptibility

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Abstract

Whole exome sequencing (WES) was used to determine the primary cause of muscle disorder in a family diagnosed with a mild, undetermined myopathy and malignant hyperthermia (MH) susceptibility (MHS). WES revealed the compound heterozygous mutations, p.Ile235Asn and p.Glu982Lys, in *ATP2A1*, encoding the sarco(endo)plasmic reticulum Ca^{2+} ATPase type 1 (SERCA1), a calcium pump, expressed in fast-twitch muscles. Recessive mutations in *ATP2A1* are known to cause Brody myopathy, a rare muscle disorder characterized by exercise-induced impairment of muscle relaxation and stiffness. Analyses of affected muscles showed the absence of SERCA1, but SERCA2 upregulation in slow and fast myofibers, suggesting a compensatory mechanism that partially restores the diminished Ca^{2+} transport in Brody myopathy. This compensatory adaptation to the lack of SERCA1 Ca^{2+} pumping activity within the muscle explains, in part, the mild course of disease in our patient. Diagnosis of MHS in this family was secondary to a loss of SERCA1 due to disease-associated mutations. Although there are obvious differences in clinical expression and molecular mechanisms between MH and Brody myopathy, a feature common to both conditions is elevated myoplasmic Ca^{2+} content. Prolonged intracellular Ca^{2+} elevation is likely to have led to MHS diagnosis in vitro and postoperative MH-like symptoms in Brody patient.

Introduction

Skeletal muscle contraction and relaxation are tightly regulated by the myoplasmic calcium (Ca^{2+}) level. Ca^{2+} released from the sarcoplasmic reticulum (SR) through the skeletal muscle Ca^{2+} release channel ryanodine receptor type 1 (RYR1) binds directly to troponin C in thin filaments, forming the "on" signal for induction of muscle contraction (MacLennan 2000). The "off" signal, that induces muscle relaxation, is the pumping of Ca^{2+} from the myoplasm back into the SR by sarco/endoplasmic Ca^{2+} ATPases (SERCA pumps). Ca^{2+} reuptake creates a Ca^{2+} store in the lumen of SR for cyclical Ca^{2+} signaling in the myofibers. Two SERCA isoforms are expressed

in mammalian muscles: sarco(endo)plasmic reticulum Ca^{2+} ATPase type 1 (SERCA1) is the major isoform in fast-twitch (type 2) skeletal muscle fibers, whereas SERCA2 is predominantly expressed in cardiac and slow-twitch (type 1) skeletal muscle fibers (MacLennan 2000; Brini and Carafoli 2009).

Mutations in the genes encoding RYR1 (*RYR1*) and SERCA1 (*ATP2A1*) are known to cause abnormal Ca^{2+} regulation: *RYR1* mutations cause malignant hyperthermia (MH) (MIM: 145600); *ATP2A1* mutations cause Brody myopathy (MIM: 601003) (Odermatt et al. 1996; Rosenberg et al. 1997–2012; Maclennan and Zvaritch 2011). These two muscle disorders differ in their clinical presentation, inheritance pattern, and prevalence. MH typically manifests as a drug-induced, severe metabolic reaction in susceptible individuals upon administration of potent inhalation anesthetics and/or depolarizing muscle relaxants. MH susceptibility (MHS) is inherited as an autosomal dominant trait with reports of over 500 different causal *RYR1* mutations (Rosenberg et al. 1997–2012; Maclennan and Zvaritch 2011). Most MHS individuals are asymptomatic and lack clinical symptoms until challenged by anesthetics. Brody myopathy is an autosomal recessive disorder, characterized by muscle stiffness, cramps, with and without pain, and progressive impairment of muscle relaxation during exercise (Brody 1969; Odermatt et al. 1996; Voermans et al. 2012). Brody myopathy is extremely rare; to date, only eight families with causal *ATP2A1* mutations have been reported (Odermatt et al. 1996; Odermatt et al. 2000; Vattemi et al. 2010; Voermans et al. 2012; Guglielmi et al. 2013).

In this article, we describe two siblings with new compound heterozygous mutations in *ATP2A1*. The index case of the family was initially referred for genetic analysis of MH due to postoperative rigidity; subsequent caffeine-halothane contracture tests (CHCT) were positive for MHS. We present clinical, genetic, and biochemical evidence that the siblings were suffering from Brody myopathy. Protein analyses showed near absence of SERCA1 and a significant twofold increase in SERCA2 content in affected muscle that was confirmed by immunofluorescent confocal microscopy showing SERCA2 upregulation in both slow- and fast-twitch fibers, providing the first clear evidence of a compensatory mechanism that partially restores diminished Ca^{2+} transport in Brody myopathy. This compensatory adaptation to the lack of SERCA1 Ca^{2+} pumping activity within the muscle explains the mild course of disease in our patients. MHS diagnosis in this family was secondary to a loss of SERCA1 due to disease-associated mutations. We discuss the relationship between Brody myopathy and MHS, emphasizing challenges in clinical and molecular genetic diagnostics.

Material and Methods

The study was approved by the Institutional Review Boards of the participating institutes. After obtaining informed consents, the index case and his sister were enrolled in the genetic study.

Patients and muscle biopsy

A 67-year-old male patient with a history of muscle pain, weakness, heat intolerance, and inability to run had cardiac surgery with no adverse reaction to anesthetics. However, he developed skeletal muscle rigidity after an operation and his creatine kinase level was slightly elevated. The patient's sister complained of muscle weakness and pain, inability to run, and considered herself and her brother to have been "handicapped" since childhood. She was prescribed a muscle relaxant, which improved her symptoms. She did not complain of heat intolerance. She has had three children who are all able to run and participate in sports. She stated that her parents were farmers and did not have any muscle symptoms.

Biopsies were taken from the following muscles: for index case *vastus lateralis* and for control individuals *gracilis*, and stored at −70°C. Prior to freezing, the index case muscle biopsy was kept in Ringer solution during the whole time of CHCT procedure, up to 3 h at room temperature (RT). For consistency, control muscle samples were collected from biopsy specimen kept under similar conditions, unless otherwise specified. Control individuals (n = 4) were unrelated healthy adult males that were diagnosed MH negative (MHN) by CHCT.

Histopathology and muscle contracture test

The muscle pathology report of the index case showed an increase in the proportion of fibers containing internal nuclei. Necrotic or regenerating fibers were not observed. Inflammatory changes were absent. There was an increase in connective tissue endomysial and perimysial spaces. In muscle sections stained for NADH dehydrogenase activity, a few fibers exhibited decreased enzyme activity near their centers. Scattered cytochrome c oxidase negative fibers were present. In sections stained for ATPase, type 2A fibers were smaller in mean diameter and essentially all of the atrophic fibers were histochemically type 2A. The patient was diagnosed with mild, undetermined myopathy. Because of postoperative rigidity, the patient was referred for MHS testing, which was performed according to the North American CHCT protocol (Larach 1989). The maximum increases from baseline tensions were 5.74

and 1.31 g to 3% halothane and to 2 mmol/L caffeine, respectively, and the patient was diagnosed as MHS. There was no family history of anesthetic complications, and the subject had three uneventful surgeries under general anesthesia prior to his heart surgery. All of his siblings, including the affected sister, had received general anesthesia without complications.

Genetic analysis

Mutation screening of RYR1 and ATP2A1

Genomic DNA (gDNA) was extracted from peripheral blood and saliva using standard protocols. Complete sequencing of *RYR1* was performed in the index case. A cohort of 50 independent subjects diagnosed as MHS by positive contracture test results was screened for the entire ATPase, Ca^{2+}-transporting, fast-twitch 1 (*ATP2A1*; MIM: 108730) gene. Primers used for screening of the entire *RYR1* were published previously (Groom et al. 2011). Primers for screening of *ATP2A1* exons were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and available upon request. Both genes were screened by Sanger sequencing and results were analyzed by Sequence Scanner Software (ABI, v.1.0) and Basic Local Alignment Search Tool (BLAST, NCBI, NIH, Bethesda, MD). The p.Lys1393Arg mutation in exon 29 of *RYR1* was screened in a cohort of 107 independent subjects diagnosed as MHN. Mutation analysis was performed by digesting amplified exon with restriction enzyme BstNI, followed by validation with Sanger sequencing.

Whole exome sequencing

The gDNA sample from the index case was subjected to whole exome sequencing (WES). WES was performed at Edgebio (EgdeBio, Gaithersburg, MD). The analysis framework to select missense single nucleotide polymorphisms and coding insertions/deletions that are not present in publicly available exome databases and to determine variant impact was used as described (Sambuughin et al. 2013; Toro et al. 2013). Mutation impact was determined with PolyPhen, SIFT, and Mutation Accessor. Novel mutations identified by WES were validated with standard Sanger sequencing of amplified DNA fragments in both affected siblings.

Segregation analysis of the Ile235Thr and Glu982Lys mutations in APT2A1

A 2386 bp fragment of the human *ATP2A1* transcript encoding SERCA1 amino acids 235–982 was generated using skeletal muscle mRNA from the index case. Reverse transcription was conducted with forward primer: 5′-ACCAGGACAAGAAGAACATGC-3′; reverse primer: 5′-TAGGTAGTTCCGAGCAACGAA-3′, using SuperScript Reverse Transcriptase (Life Technologies, Grand Island, NY), followed by PCR reactions with long fragment DNA polymerase (Takara, Mountain View, CA). PCR products were then subcloned directly into competent cells using the TOPO-TA cloning kit (Life Technologies). Plasmid DNA from 16 colonies was sequenced, 13 contained the sequence encoding amino acids 235–982. Among analyzed colonies, eight contained the sequence encoding the lle235Asn mutation and five contained the sequence encoding the Glu982Lys mutation. No colonies were found to contain plasmid DNA with both mutations and none contained the wild-type sequence only.

Screening of RYR1 transcript

The entire *RYR1* transcript was generated using skeletal muscle mRNA extracted from the index case. Reverse transcription reactions to synthesize cDNA were described in the previous section. Overlapping cDNA fragments were amplified and sequenced using Sanger sequencing.

Protein analysis

SDS-gel electrophoresis and Western blot analysis of protein in whole-muscle homogenates were performed as described in (Kraeva et al. 2013) with modifications. Briefly, frozen muscle samples (~150 mg) were powdered under liquid nitrogen and homogenized in 0.6-mL ice-cold buffer, containing 50 mmol/L Tris-HCl (pH 7.4); 10 mmol/L EGTA; 2 mmol/L EDTA; 5 mmol/L DTT; 0.5 mmol/L PMSF, and protease inhibitor cocktail (Complete mini, Roche Diagnostics, Indianapolis, IN). Muscle homogenates (0.5–20 μg total protein) were mixed with 2x SDS sample buffer and boiled for 5 min prior to protein separation on 5–15% Mini-PROTEAN TGX™ SDS-PAGE gels (Bio-Rad Laboratories, Mississauga, ON, Canada). BLUeye Prestained Protein Ladder (GeneDireX, Atlanta, GA) was used as a protein molecular weight marker. Following electrophoresis, the proteins were transferred onto Immobilon P^{SQ} membranes (0.2-μm pore size; EMD Millipore, Billerica, MA). The membranes were blocked with 5% (w/v) nonfat dry milk in 10 mmol/L PBS, pH 7.2, for 1 h at RT and incubated with one of the following mouse monoclonal IgG antibodies: anti-panRYR (1:250; 34°C, Thermo Scientific, Rockford, IL) and anti-RYR1 (1:1000, XA7B6; Upstate Cell Signaling Solutions, Lake Placid, NY); anti-SERCA1 (1:24,000, VE121G9; Abcam, Toronto, ON, Canada) and anti-SERCA2 (1:20,000, IID8; Sigma,

Oakville, ON, Canada). SERCA1 protein expression was also tested with polyclonal goat anti-human SERCA1 antibody, N-19 (1:200; Santa Cruz Biotechnology, Dallas, TX) and with mouse monoclonal antibody A52 (Zubrzycka-Gaarn et al. 1984).

Following incubation with the corresponding, anti-mouse IgG (1:1000; Sigma-Aldrich, Oakville, ON, Canada) and anti-goat IgG (1:1000; Sigma) antibodies, conjugated to horseradish peroxidase. The immune complexes were revealed using chemiluminescent Luminata™ Forte Western HRP substrate (EMD Millipore). For loading controls, the blots were reprobed with at least one of the following antibodies: mouse monoclonal anti-α-tubulin IgG (1/200, 6A204; Santa Cruz Biotechnology), mouse monoclonal anti-α-actin IgM (1:60,000, 5C5; Sigma), and rabbit polyclonal anti-GAPDH (1:3000; Millipore). The images were generated using Fluo S™ Max MultiImager and Quantity One software (Bio-Rad Laboratories). Densitometry was performed with the ImageJ software (National Institute of Health, Bethesda, MD).

Confocal microscopy

Longitudinal and transverse cryostat sections (7 μm) of control and Brody patient skeletal muscle biopsy were fixed in 4% PBS buffered formaldehyde, 5 min at RT. After blocking for 1 h at RT in PBS, containing 10% chicken serum (Life Technologies), 0.1% Triton X100 (Sigma), and 1% BSA (Bio-Shop, Canada Inc), the sections were incubated with mouse monoclonal antibodies against fast- and slow-twitch skeletal muscle myosins (1/200; Sigma) and SERCA2 (1:50, Sigma) overnight at 4°C. For visualization of connective tissue, the sections were costained with wheat germ agglutinin (WGA) Alexa Fluor 633 conjugate (WGA-Alexa, 1 μg/mL; Life Technologies). Sections were then washed (4 × 5 min) in PBS, containing 0.05% Tween 20 (PBS-T; Sigma) and incubated with secondary antibodies – chicken anti-mouse IgG1 Alexa Fluor 488 (1:1000; Life Technologies) for 1 h at RT. Sections were washed (3 × 5 min) in PBS-T and mounted with anti-fade UltraCruz Mounting media containing DAPI (Santa Cruz) and viewed under Olympus Fluoview B a laser scanner microscope. The images were analyzed using Olympus FV100 version 3.1 image viewer software (Olympus, Center Valley, PA).

Data analysis and statistics

The results are expressed as mean ± SEM obtained from multiple determinations in at least three separate experiments. The significance of changes was evaluated using the unpaired Student's *t*-test. The $P < 0.05$ was considered significant.

Results

Genetic analysis and whole exome results

The MHS diagnosis for the index patient prompted us to search for a mutation in *RYR1*. Genetic analysis of both the index case and his affected sister identified a heterozygous c.4178A>G; p.Lys1393Arg mutation in exon 29 of *RYR1* (NM_001042723) (Fig. 1A). The p.Lys1393Arg mutation was reported previously in association with MHS, exertional rhabdomyolysis, and axial myopathy (Broman et al. 2009; Dlamini et al. 2013; Løseth et al. 2013). The p.Lys1393Arg mutation is also reported in the general population at a frequency ≥1% (Broman et al. 2009; Gonsalves et al. 2013; Exome Variant Server at http://evs.gs.washington.edu/EVS/), which is well above the estimated frequency of MHS-associated mutations in *RYR1* (Rosenberg et al. 1997–2012; Brandom et al. 2013). Because the general population cannot truly present the MHN control population, we have screened 107 subjects with MHN diagnosis for the p.Lys1393Arg mutation. The results revealed that three MHN controls carried the mutation, suggesting that the association of the p.Lys1393Arg mutation with MHS is weak. Of most importance in considering the association of this mutation with disease in our family, the highly penetrant childhood onset of muscle disease in two affected siblings, with no history of muscle disease in either parent, cannot be explained by the presence of a familial, dominantly inherited *RYR1* mutation. A deep intronic mutation causing faulty splicing has been found in a RYR1-related myopathy and these types of mutations can be missed by gDNA screening (Monnier et al. 2008). To determine whether any such changes might have been missed during gDNA screening, the *RYR1* transcript was also analyzed, the results of the entire *RYR1* transcript analysis were negative.

To identify the true underlying cause of the myopathy, we turned to WES. WES was performed on gDNA from the index case, which resulted in about 87% of the targets reaching >20× coverage. WES analysis confirmed the presence of the p.Lys1393Arg in *RYR1* and showed negative results for other MH causative gene, *CACNA1S* (Rosenberg et al. 1997–2012; Maclennan and Zvaritch 2011), encoding the α1-subunit of the skeletal muscle Ca^{2+} channel and *CASQ1* encoding calsequestrin, a causal factor of an MH-type reaction in mice (Dainese et al. 2009). A new, c. 2974C>T, p.His992Tyr, mutation was detected in *CACNA2D1* (NM_000722). Although this gene encodes the α2/∂ subunit of the skeletal muscle Ca^{2+} channel and is located within a chromosomal region linked to MHS (Iles et al. 1994; Robinson et al. 2003), a causal role for this mutation was ruled out on the basis of its predicted

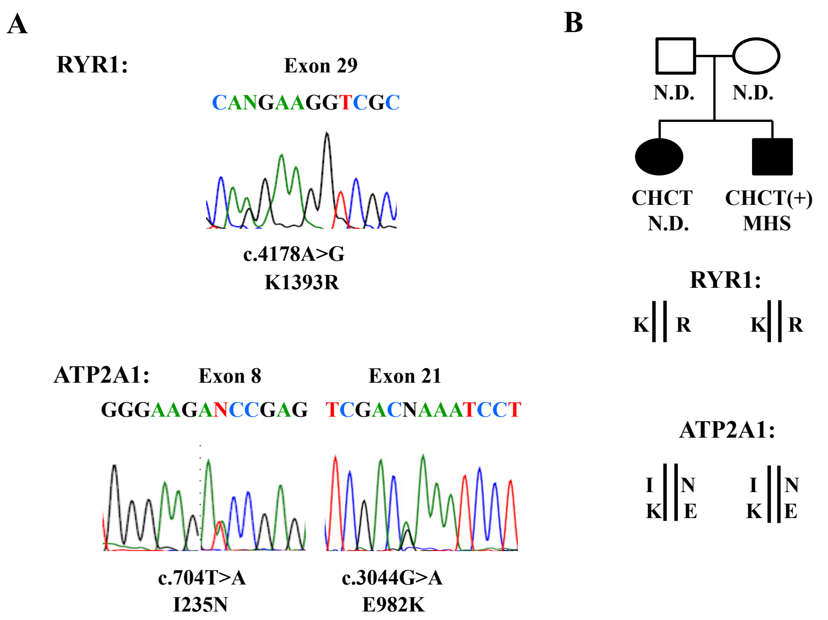

Figure 1. (A) Sequence chromatographs of *RYR1* and *ATP2A1* in the index case. Positions of nucleotide changes (N) those result in p.Lys1393Arg (K1393R) in *RYR1* and p.Ile235Asn (I235N) and p.Glu982Lys (E982K) in *APT2A1*. (B) Segregation of alleles in the affected siblings. Filled symbols indicate affected individuals. CHCT denotes caffeine and halothane contracture test. N.D. indicates not determined. Empty symbols indicate clinically healthy parents.

dominance and its predicted benign effect, but mainly on the basis of its absence in the affected sibling.

Among additional novel missense mutations identified in the index case, we considered c.2125G>T, p.Gly709Stop mutation in the *KIAA0196* (NM_014846) encoding strumpellin as potentially significant, since this mutation was shared by both affected siblings. It introduced a stop codon mutation that would likely lead to protein truncation. Dominant mutations in *KIAA0196* are associated with an aggressive subtype of spastic paraplegia, SPG8 (Valdmanis et al. 2007), characterized by spastic gait, spasticity of lower limbs, hyperreflexia, and decreased vibration sensation, with an age of onset between 20 and 30 years. None of these symptoms corresponded to the symptoms of our two affected siblings, who exhibited awkwardness and exercise-induced contractures in their earliest childhood. In addition, all known disease-associated *KIAA0196* mutations are missense and no truncating mutations have been reported in SPG8 patients, suggesting that gain of function rather than loss of function mutations is a highly relevant feature of the pathogenesis of this disorder. Therefore, an association of the *KIAA0196* p.Gly709Stop with myopathy in our family was ruled out.

We identified two new heterozygous missense mutations, c.704T>A; p.Ile235Asn in exon 8 and c.2944G>A; p. Glu982Lys in exon 21, in *ATP2A1* (NM_004320) (Fig. 1A). These mutations have not been reported in ClinSeq, 1000 genomes databases, or the publicly available database of >6500 European and African American exomes (Exome Variant Server: http://evs.gs.washington.edu/EVS/). The two mutations were confirmed by Sanger sequencing in both the index case and his affected sister. Both mutations were predicted to have damaging effects by three different predictive programs.

To characterize how these two mutations segregate within our family, we cloned *ATP2A1* transcripts isolated from the index case. Sequencing of *ATP2A1* clones showed that p.Ile235Asn and p.Glu982Lys occurred as compound heterozygotes, inherited in a recessive fashion (Fig. 1B). The inheritance of recessive mutations in *ATP2A1* has been shown previously to cause Brody myopathy, which presented in childhood and caused exercise-induced impairment of muscle relaxation and stiffness with and without muscle weakness and pain (Brody 1969; Voermans et al. 2012). These are the signs and symptoms that characterize the clinical features of our affected siblings. Because the index case was diagnosed with MHS, we screened the entire *ATP2A1* in 50 independent MHS subjects. No significant *ATP2A1* mutation was identified in any of these patients (Table S1).

Mutation localizations in the SERCA1

The SERCA1 protein consists of a single polypeptide chain folded into three major domains: actuator (A); nucleotide binding (N); phosphorylation (P); and 10 transmembrane (TM) helices (MacLennan et al. 1998; Brini and Carafoli 2009). Ile 235 and Glu 982 are highly conserved in SERCA1 throughout different species (data not shown). Ile 235 is located in the A domain where the majority of *ATP2A1* mutations associated with Brody myopathy have been found (Fig. S1). Ile 235 lies at the beginning of the $\alpha 3$ helix of the A domain (Fig. 2), where a change from a

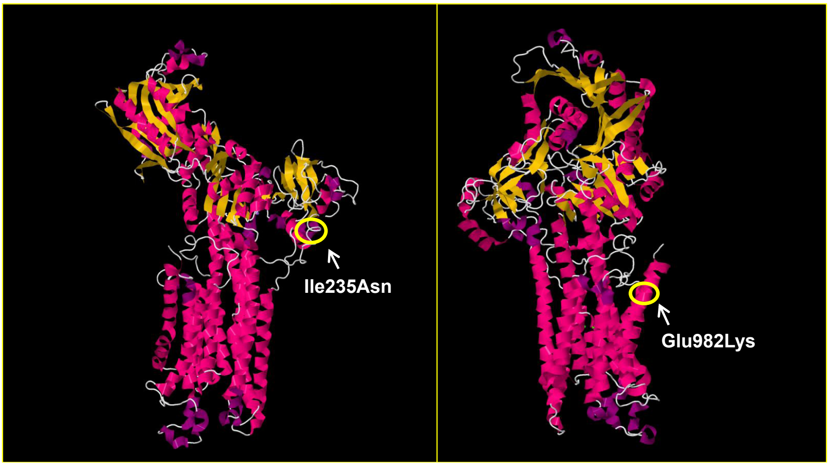

Figure 2. Disease-associated mutations in the Ca^{2+} bound rabbit sarco(endo)plasmic reticulum Ca^{2+} ATPase type 1 crystal structure (1SU4P) from the Protein Data Bank (www.rcsb.org/pdb). The positions of two new mutations identified in this study are circled.

nonpolar isoleucine to a polar asparagine may have a destabilizing effect on SERCA1 function. Glu 982 lies within the last TM domain (TM10) of the protein (Fig. 2). In this position, a change from a negatively charged glutamate to a positively charged lysine is also likely to have a damaging effect on the stability of SERCA1.

Protein analysis

To determine the effect of *ATP2A1* mutations on muscle protein integrity, we analyzed the expression of SERCA1 and related proteins in skeletal muscle biopsies from the index case and healthy adult male subjects (n = 4) diagnosed as MHN by MH contracture tests. Western blot analysis of whole-muscle protein extracts with a monoclonal anti-SERCA1 antibody VE121G9 revealed a dramatic decrease in SERCA1 protein content in our Brody patient muscle (Fig. 3A). Densitometric analysis of semiquantitative Western blots showed a 20-fold (19.6 ± 3.3, n = 6, P < 0.05) reduction in SERCA1 protein in the muscle of our Brody patient (Fig. 3B). The near absence of SERCA1 was further confirmed by Western blot analysis using two additional SERCA1-specific antibodies: a mouse monoclonal, A52; and goat polyclonal antibody, N-19, directed against the *N*-terminal, human-specific SERCA1 peptide (Fig. 3C). Neither of these antibodies revealed any appreciable amounts of the full-length SERCA1 protein or any products of SERCA1 fragmentation in the broad range of the polypeptide mass from 110 to 10 kDa, arguing against SERCA1 degradation following muscle harvesting as a possible cause of its absence.

The SERCA2 level in our Brody patient muscle, compared to control muscle samples, was estimated relative to a set of loading control proteins: α-actin, α-tubulin, GAPDH, and HPRT, through semiquantitative Western blot analysis (Fig. 4A). Relative estimates using different loading controls produced essentially the same results, indicating a significant, almost twofold increase in SERCA2 expression levels (1.8 ± 0.24, n = 6, P < 0.05), compared to control muscle samples.

Western blot analysis for RYR1 was performed using 34C pan-RYR antibody with an epitope mapped to amino acids 2756–2803 of the human RYR1 sequence (Meng et al. 2007). Surprisingly, the full-length RYR1 band that was readily detectable in control samples was not observed in the Brody patient muscle sample (Fig. 4B). Instead, two major RYR1-specific protein bands of slightly lower molecular mass, estimated to be 350 and 420 kDa were detected. In addition, staining with anti-RYR1 antibody XA7 (Paul-Pletzer et al. 2005) directed against amino acids 590–609 in the *N*-terminal portion of the protein showed a single immunoreactive band of about 80 kDa (Fig. 4C) that was also present in much lower amounts in control muscle samples.

The finding of RYR1 proteolysis in our patient is inconsistent with the fact that there was no obvious impairment of excitation–contraction coupling in either of the two siblings, which would imply that RYR1 had been fully functional throughout their lives. The combined antibody staining density revealed roughly the same amount of total RYR-specific immunoreactive material in the muscle samples of the index patient and control subjects. This suggests that proteolysis observed in the muscle samples from our Brody patient took place upon muscle harvesting and was limited to as few as two sites near the *N*-terminal end of the RYR1 polypeptide. This proteolysis appeared to be specific for RYR1, since we did not observe any proteolysis of SERCA2 or any of the loading control proteins (Fig. 4).

Ryanodine receptor type 1 protein is known to be susceptible to partial proteolysis by Ca^{2+}-dependent proteases such as calpain upon skeletal muscle sample excision and handling (Wu et al. 1997; Tompa et al. 2004), but in all cases reported the full-length RYR1 protein has been

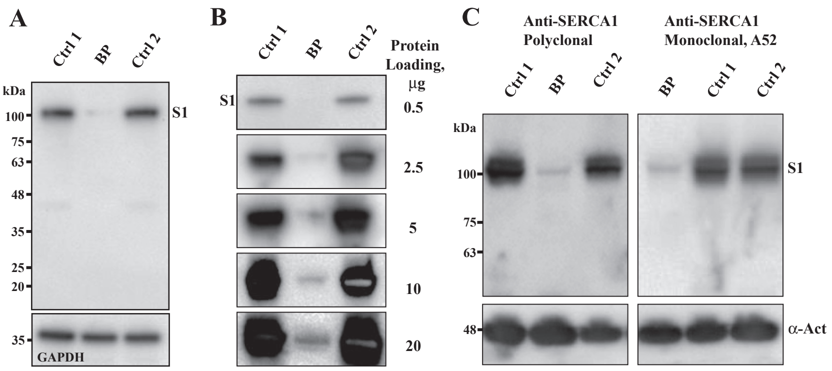

Figure 3. Western blot analysis of sarco(endo)plasmic reticulum Ca^{2+} ATPase type 1 (SERCA1). Ctrl, control samples; BD, patient sample. (A) Almost complete absence of SERCA1 (S1) was revealed in Brody patient compared to control samples. (B) Semiquantitative analysis of SERCA1 protein expression using mouse monoclonal IID8 anti-SERCA1 antibodies. The amounts of total loaded protein in the muscle homogenates are indicated on the right. In the Brody muscle, a well-defined SERCA1 protein band is revealed only at high protein loadings of 10 and 20 μg protein. At similar loadings, control samples show overloaded and oversaturated SERCA1 protein bands. (C) Analysis of muscle homogenates using goat polyclonal (left panel) and mouse monoclonal A52 (right panel) anti-SERCA1 antibodies.

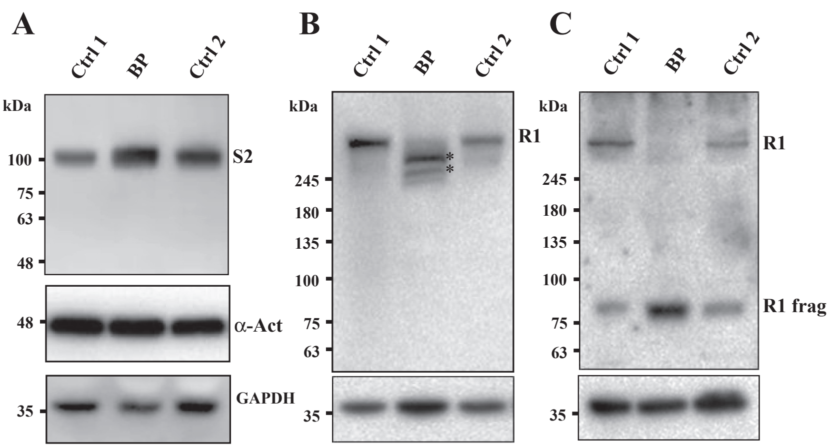

Figure 4. Western blot analysis of sarco(endo)plasmic reticulum Ca^{2+} ATPase type 2 (SERCA2) and ryanodine receptor type 1 (RYR1) expression in skeletal muscle biopsy of the Brody patient (BP) and two healthy control individuals (Ctrl1 and 2). One (A) and 10 mg (B–C) total protein from whole-muscle homogenates were resolved on 4–15% gradient SDS-gels. (A) SERCA2 (S2) protein expression is increased almost twofold in the BP muscle. (B) Anti-RYR 34C antibodies fail to detect the full-length RYR1 (R1) protein in the BP muscle but reveal well-defined polypeptide bands of a lower molecular mass (asterisks) indicating RYR1 proteolysis. (C) Anti-RYR1 XA7 antibodies fail to reveal the full-length RYR1, but detect a lower molecular mass fragment of about 80 kDa (R1 frag). Immunodetected bands of α-actin (A) and GAPDH (A–C) were used as loading controls. Molecular mass standards are indicated on the left of each panel.

present as the major immunoreactive band in Western blots. The muscle sample from our index patient was from a muscle biopsy used in CHCT analysis. Prior to freezing, the sample was kept for about 3 h at RT in oxygenated Ringer solution. To test whether complete loss of the full-length RYR1 protein could be reproduced in control MHN muscle samples, incubated for a prolonged period at RT in Ringer solution or exposed to the CHCT test, we performed Western blot analysis of MHN control muscle samples exposed to these conditions, which might have evoked RYR1 proteolysis (Fig. S2). We observed some proteolysis of RYR1, but not the complete disappearance of the full-length RYR1 protein in these control samples. Thus, we concluded that extensive RYR1 proteolysis observed in the Brody patient muscle was specific to RYR1 itself and, moreover, that it was specific to the conditions existing in the patient's muscle. We do not think that it was related to the heterozygous presence of the p.Lys1393Arg mutation, since this should affect only 50% of the RYR1 protein. However, tetramer formation might have unanticipated effects on proteolytic sensitivity. A more attractive alternate possibility is that prolonged elevation of Ca^{2+} in the muscle, resulting from the loss of SERCA activity might have activated a Ca^{2+}-dependent

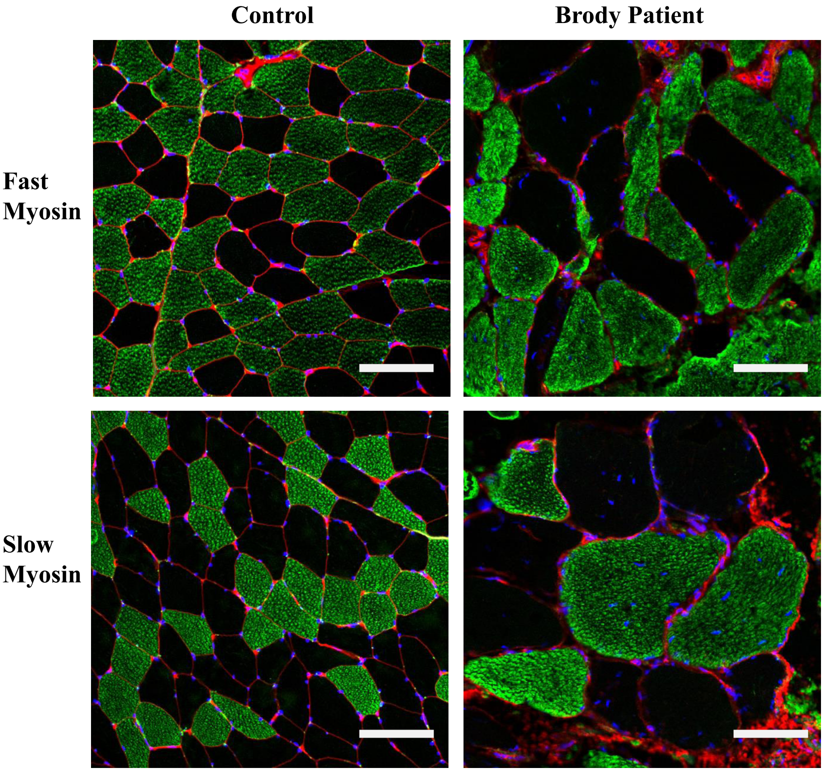

Figure 5. Immunofluorescence staining for fast and slow myosins in the muscle of Brody patient (BP). Confocal microscopy images of transverse skeletal muscle sections stained with anti-fast (top) and anti-slow (bottom) myosin antibodies (green). WGA (red) stains connective tissue. DAPI (blue) counterstains nuclei. Increased fiber size variability and fiber hypertrophy is readily observed in both types of the BP muscle fibers. Bar, 100 μm.

protease, such as calpain, that would degrade RYR1 with the high specificity that we observed.

Confocal microscopy

We addressed whether SERCA2 upregulation occurred in fast fibers that lack SERCA1 or in slow fibers that express SERCA2 normally by immunostaining of affected muscle. Immunofluorescence staining of our Brody patient muscle sections for slow- and fast-twitch myosins confirmed previous histological findings of marked variation in fiber size and increased number of internal nuclei (Fig. 5). Both types of fibers exhibited hypertrophy, but it was most prominent in slow fibers, the mean diameter being three- to fourfold greater than those in controls. Fast fibers showed high-fiber size variability and up to 90% of them had multiple, centrally located nuclei that were abnormally elongated. Immunofluorescence staining of our Brody patient muscle sections with anti-SERCA2 antibodies revealed higher than normal immunoreactivity in both fast and slow fibers (Fig. 6A). We observed a 1.5- to twofold increase in SERCA2-specific immunoreactivity in Brody slow fibers compared to control slow fibers, whereas the SERCA2-specific immunostaining within the Brody fast fibers showed a significant fourfold increase compared to the control fast fibers (Fig. 6B). The elevated SERCA2 expression in fast fibers lacking SERCA1 supports the idea that a compensatory mechanism is activated in these myofibers, representing a novel finding in Brody disease pathology.

Discussion

We describe a family in which two affected siblings presented with childhood onset exercise intolerance, including the inability to perform repeated tasks such as running, muscle pain, and weakness. The parents and children of the siblings were healthy, suggesting that inheritance of the myopathy was recessive. There was no familial history of any anesthetic complications, but the proband developed postoperative rigidity followed by a positive MH contracture test, which resulted in his diagnosis as MHS. Candidate gene screen, followed by exome analysis, revealed that the proband and his affected sister inherited mutations in two genes that have been associated with two different muscle diseases. A search of *RYR1* that is associated with the majority of dominantly inherited MH cases revealed the presence of a heterozygous p.Lys1393Arg mutation in both affected siblings. We have ruled out the possibility that this mutation is the cause of myopathy in the affected siblings, since the muscle disease observed in our family was inherited recessively.

Whole exome sequencing revealed the presence of two recessively inherited compound heterozygous mutations

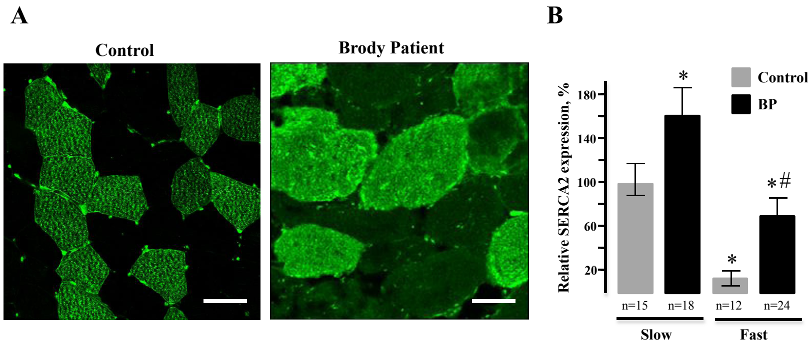

Figure 6. Analysis of sarco(endo)plasmic reticulum Ca^{2+} ATPase type 2 (SERCA2) immunoreactivity in myofibers of Brody patient (BP). (A) Representative immunofluorescence confocal microscopy images of transverse cryostat sections from a control (left) and our BP (right) skeletal muscle biopsy stained with anti-SERCA2 antibodies (green). The sections were processed in parallel and the images were taken at identical microscope and laser intensity settings. The control muscle shows a characteristic pattern of SERCA2 expression that is restricted to slow-type myofibers, while the fast-type myofibers that do not express SERCA2 remain unstained and appear dark. In the muscle section from our BP (right), SERCA2 immunoreactivity is detected in both fast and slow myofibers. Bar, 50 μm. (B) Semiquantitative analysis of SERCA2-specific immunofluorescence reactivity in fast- and slow-type myofibers of the control and BP muscles relative to SERCA2 reactivity in the control slow myofibers. Vertical bars represent standard error of the mean. The number of samples (*n*) is the number of fibers assessed for each group. *Significantly different compared with control slow fibers ($P < 0.05$). #Significantly different compared with control fast fibers ($P < 0.05$).

in *ATP2A1* that would encode for p.Ile235Asn and p.Glu982Lys in SERCA1. Both of these *ATP2A1* mutations are newly identified in this study and are absent in >6500 exomes. They change highly conserved residues in SERCA1, with effects that are predicted by various analyses to be damaging. The locations of both mutations in the protein structure show that p.Ile235Asn is likely to disturb movements of the A domain, whereas p.Glu982Lys may interfere with the function of transmembrane helix TM10 of SERCA1. Protein analyses of affected muscles showed the absence of SERCA1. These results demonstrate that p.Ile235Asn and p.Glu982Lys mutations are disease associated and that the siblings carrying these mutations suffer from Brody myopathy.

A severe reduction in SERCA1 expression and SERCA1 activity has been reported for many Brody patients carrying a number of SERCA1 mutations (Brody 1969; Karpati et al. 1986; Odermatt et al. 2000; Vattemi et al. 2010; Guglielmi et al. 2013). The reduction in SERCA1 expression in Brody patients ranges from 50% to complete loss of the protein. Remarkably, patients, including ours, tolerate this near absence of SERCA1 and they are still able to relax their muscle, albeit at very slow rate. Thus, Brody disease has a mild presentation when muscular activity is paced at tolerable levels, but becomes acute with rapid, repetitive exercise. This suggests the presence of a compensatory Ca^{2+} removal by other functionally similar proteins, including plasma membrane Ca^{2+} ATPases, SERCA2, and SERCA3 (MacLennan et al. 1998; MacLennan 2000). Among these, SERCA2 is likely to play the most significant role in skeletal muscle Ca^{2+} transport. Indeed, SERCA2 expression was increased by approximately twofold in our patient. This upregulation was independently confirmed by confocal microscopy. Immunostaining of affected muscles revealed 2–4 times higher than normal levels SERCA2 staining in both slow and fast myofibers, respectively. The elevated SERCA2 expression in fast fibers lacking SERCA1 clearly demonstrate a compensatory mechanism that partially restores diminished Ca^{2+} transport in Brody patients that are heavily muscled. This might be explained by marked hypertrophy of slow-twitch muscles due to the effort involved in trying to force relaxation on fast-twitch fibers, with little capacity to pump Ca^{2+}, by compensatory activation of SERCA2.

Since our affected siblings shared disease-associated mutations in *RYR1* and *ATP2A1*, and the index case was diagnosed as MHS after postoperative rigidity, we considered the possibility that this family is affected with two different coexisting diseases, MH and Brody myopathy. It is well established that pathogenic dominant *RYR1* mutations, when exposed to triggering conditions (anesthetics in humans; stress in swine), induce spontaneous, oscillating Ca^{2+} release that rapidly elevates myoplasmic Ca^{2+} levels, leading to a fulminant MH reaction (Rosenberg et al. 1997–2012; Maclennan and Zvaritch 2011). Such results have been demonstrated with the p.Lys1393Arg mutation expressed in human B cells (Vukcevic et al. 2010), but the larger body of evidence for a role for this mutation in MH in humans is not strongly supportive. This mutation occurs in the general and MHN control populations at a frequency of 1–3% which is well above the estimated frequency of MHS-associated mutations in *RYR1* (Rosenberg et al. 1997–2012; Brandom et al. 2013). There is no clear evidence of segregation of this mutation with MHS phe-

notype in affected families (Broman et al. 2009; Dlamini et al. 2013; Løseth et al. 2013). The mutation is also predicted to have a negligible impact on protein function by mutation prediction analyses. All of these data, contrary to published reports, indicate that the p.Lys1393Arg is not a pathogenic disease causing mutation; it should be classified as a benign polymorphism. Recent analyses of 850 exomes indicate that a number of *RYR1* polymorphisms have been misclassified as pathogenic mutations in early MH genetic studies (Gonsalves et al. 2013).

We have observed a limited proteolysis of RYR1 that appears to be specific for the RYR1 protein in our Brody family and may result from unrecognized consequences of mutant SERCA1. Depletion of SR (and endoplasmic reticulum [ER]) Ca^{2+} due to mutant SERCA1 could lead to an ER stress response leading to protein aggregation and degradation similar to that evoked by mutations in SERCA2 (Ahn et al. 2003; Wang et al. 2011). However, ER stress leads to progressive disease. The mild presentation of myopathy and a lack of progression of Brody disease would not be expected if an ER stress response were triggered, leading to apoptosis, as is possibly the case in progressive congenital myopathies arising from recessive mutations in *RYR1* (Zvaritch et al. 2009; Maclennan and Zvaritch 2011). Proteolysis of RYR1 likely occurred after the harvest of the tissue. In keeping with this view, there was no obvious impairment of excitation–contraction coupling in studied patients, indicating that protein was fully functional throughout their lives.

It has been reported that some of the Brody patients developed MH symptoms or have been diagnosed as MHS by contracture testing (Karpati et al. 1986; Odermatt et al. 1997; Odermatt et al. 2000; Guglielmi et al. 2013). However, MH symptoms in our Brody patient developed only postoperatively, which is not a common feature of a typical MH episode. While postoperative complications have been reported in some MH patients (Burkman et al. 2007; Larach et al. 2008), MH episode usually develops within the first few hours of exposure to inhalation anesthetics with signs of hypercarbia, tachycardia, and rapid increase in temperature (Burkman et al. 2007; Visoiu et al. 2014). Outside of the operating room, MH is diagnosed by muscle contracture tests (Larach 1989; Ording et al. 1997) that are highly sensitive to abnormal myoplasmic Ca^{2+} regulation. However, the sensitivity of contracture tests is compromised by its low specificity of 78% (Allen et al. 1998). MHS diagnosis in Brody myopathy patients might be due to the 22% false-positive rate of the CHCT bioassay. While there is an obvious difference in clinical presentation, molecular genetics, and disease mechanisms between MH and Brody myopathy, a feature common to both conditions is an elevated myoplasmic Ca^{2+} concentration. We suggest that the cause of the elevated myoplasmic Ca^{2+} that produced abnormal results on CHCT leading the diagnosis of MHS in our index patient was the mutations in SERCA1, not the mutation in RYR1.

In conclusion, we identified a family with Brody myopathy associated with new compound heterozygous mutations in SERCA1. Affected muscle showed near absence of SERCA1 and significant increase in SERCA2 protein level, demonstrating a mechanism that partially restores diminished Ca^{2+} transport. The index case of the family was initially referred for genetic analysis of MH due to postoperative skeletal muscle rigidity; subsequent CHCT led to diagnosis of MHS. We conclude that positive MH contracture responses in vitro or postoperative rigidity with creatine kinase elevation in our Brody patients are due to prolonged high levels of Ca^{2+} as a consequence of compound heterozygous mutations in the *ATP2A1* gene that lead to the lack of SERCA1 protein and SERCA1 Ca^{2+} pumping activity. Our finding of a compensatory SERCA2 upregulation in both slow and fast myofibers lacking SERCA1 protein is novel for Brody disease pathology and may explain a relatively mild disease phenotype developing in the absence of the canonically predominant skeletal muscle isoform – SERCA1. Finally, our work highlights the use of a comprehensive approach in elucidating the pathogenic effects of disease-associated mutations, specifically when multiple mutations are found in disease-associated genes.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Locations of *ATP1A1* mutations associated with Brody myopathy in the domain structure of SERCA1. Mutations identified in this study are underlined. The first and last amino acids of SERCA1 are in italics.

Figure S2. RYR1 proteolysis is specific to the Brody patient muscle. (A and B) Western blot analysis of RYR1 protein in the Brody patient muscle (BP) and control muscle samples exposed to various experimental conditions that could result in RYR1 proteolysis. (A) A freshly harvested control muscle sample was split into separate myofiber bundles that were frozen in liquid nitrogen immediately after harvesting (snap frozen) or after exposure to 32 mmol/L caffeine (caffeine); 3% halothane (halothane), and after a prolonged incubation for 3 h at RT in oxygenated Ringer solution, containing 2.5 mmol/L Ca^{2+} (Ringer bathed). The full-length RYR1 protein is revealed as a major band in all control muscle samples, regardless of experimental conditions. α-Actinin was used as a loading control. (B) Western blotting analysis of the Brody patient muscle sample (BP) versus a control (Ctrl) sample that has been thawed and left at room temperature for 2 h prior to analysis. The full-length RYR1 protein is readily observed in the control sample suggesting its relatively low susceptibility to proteolysis even under harsh experimental conditions.

Table S1. The *ATP2A1* polymorphisms identified in a cohort of 50 MHS subjects.

Improving Awareness of Nonanesthesia-Related Malignant Hyperthermia Presentations: A Tale of Two Brothers

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A 30-year-old man developed unexplained rhabdomyolysis, persistently increased creatine kinase and severe debilitating muscle cramps. After a nondiagnostic neurologic evaluation, he was referred for a muscle biopsy, to include histology/histochemistry, a myoglobinuria panel, and a caffeine halothane contracture test. Only the caffeine halothane contracture test was positive, and a subsequent ryanodine receptor type 1 gene evaluation revealed a mutation functionally causative for malignant hyperthermia. His identical twin brother, who was suffering from similar complaints, was found to share the same mutation. They each require oral dantrolene therapy to control symptoms, despite difficulty in identifying health care providers familiar with treating this disorder. (A&A Case Reports. 2014;3:23–6.)

Rhabdomyolysis is a relatively common condition characterized by the breakdown of skeletal muscle and the leakage of cellular constituents into the intra- and extravascular spaces after injury. A variety of factors including some prescription medications and drugs of abuse may precipitate skeletal muscle injury. Rhabdomyolysis is responsible for up to 7% of all cases of acute renal failure.[1] Exertional rhabdomyolysis (ER) occurs in response to exercise when mechanical or metabolic stress damages skeletal muscle.[2] Under extreme physical and environmental conditions, anyone can develop ER.[1] However, some individuals appear more susceptible, suggesting an underlying metabolic myopathy or genetic abnormality.[2] Malignant hyperthermia (MH) is a potentially lethal, inherited, subclinical myopathy identified by an unexpected hypermetabolic reaction during and after exposure to particular anesthetic-related medications. One clinical feature of MH is rhabdomyolysis. Both ER and MH are characterized by an uncontrolled increase in intracellular skeletal muscle calcium, leading to oxidative, chemical, and mechanical stress with irreversible muscle breakdown.[1] Mutations in the protein structure of the ryanodine receptor type I (RyR1) fast release calcium channel of skeletal muscle sarcoplasmic reticulum have been identified as the defect most strongly associated with MH susceptibility (MHS).[3] Thus, RyR1 gene (RYR1) analysis has become an invaluable diagnostic test for individuals and families with suspected MHS.

The association between MH and nonanesthesia-related ER has been a topic of debate for many years. Despite growing literature to suggest otherwise,[4–11] the concept of awake MH manifestations has been controversial and slow to gain acceptance among clinicians and scientists with MH expertise.[12] The traditional definition of MH as a pharmacogenetic disorder of skeletal muscle calcium regulation triggered by potent volatile anesthetics and/or succinylcholine[13] may describe only a portion of the clinical presentations caused by this myopathy. More accurately, MH is a subclinical metabolic myopathy that may lead to a hypermetabolic crisis on exposure to potent volatile anesthetics and/or succinylcholine,[14] and in some individuals on exposure to heat and exercise.[4–11,15–21]

Whereas unrecognized anesthetic-induced MH events that go untreated with IV dantrolene may result in >70% mortality,[22] the full extent of the awake manifestations of the syndrome are not well appreciated. RYR1 changes typical of MH may be expressed as ER, and lack of recognition of this connection may lead to patient morbidity and delay in appropriate treatment. As experts in the treatment and understanding of MH, anesthesiologists will be called on to educate and advise other caregivers in the recognition and treatment of associated myopathies having clinical manifestations similar to those of MH. In this case report, we describe the association between a RYR1 MH-causative mutation and recurrent rhabdomyolysis with and without exercise in identical male twins. Furthermore, their history demonstrates that nonanesthesiologists also require education concerning the relationship between MHS and rhabdomyolysis.

All patients described in this case report gave their consent for publication.

CASE DESCRIPTION

A 30-year-old African American man presented to the Emergency Department (ED) with a complaint of chest pain after working outside on a hot summer day. The medical

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evaluation was significant only for a creatine kinase (CK) of 3900 U/L (normal <200 U/L). Rhabdomyolysis was diagnosed, and the patient was treated with IV hydration and discharged home. His CK levels remained increased (>2000 U/L) for 2 months before gradually decreasing to the 1000 U/L range. After this episode, the patient developed persistent exercise intolerance, reporting severe muscle cramps with only moderate exertion. His symptoms progressed to include jaw and face cramps at rest and a complete inability to exercise. Of note, he had undergone 2 uncomplicated surgical procedures with general anesthesia as a child, and he denied a personal or family history of MH. The patient was referred for evaluation of myopathic disorders, including MH, under an IRB-approved protocol.

The patient underwent a complete physical examination, including electromyography, nerve conduction studies, serial CKs, plasma and urine myoglobin, urinalysis, standard chemistries, lipid panel, thyroid panel, metanephrines panel, erythrocyte sedimentation rate, antinuclear antibodies, rheumatoid factor, rapid plasma reagin, and testing for human immunodeficiency virus. An Exercise Intolerance Mutation Profile was performed to screen for the most common genetic causes of metabolic myopathies (carnitine palmitoyltransferase 2 deficiency, CPT2: S113L, 413delAG, P50H, R503C, G549D, R631C; myoadenylate deaminase deficiency, AMPD1: Q12X, P48L; myophosphorylase deficiency, PYGM: R49X, G204S). All tests were within normal limits.

The patient was referred to a MH diagnostic center for a muscle biopsy. The histology/histochemistry findings were nonspecific, and a myoglobinuria panel was negative. The caffeine halothane contracture test (CHCT) was positive for MH, with muscle contracture increases from baseline twitch tension in 3 separate muscle strips of 6.1, 2.4, and 3.8 g, respectively, to 3% halothane (positive ≥0.7 g). Another 3 separate muscle strips had contracture increases from baseline twitch tension of 0.4, 0.9, and 0.5 g, respectively, to 2 mM caffeine (positive ≥0.3 g). Targeted gene sequencing of CACNA1S and RYR1 (the 2 genes most strongly associated with MH) was performed. The CACNA1S evaluation was negative, but the RYR1 was positive for an Arg2454Cys mutation, a mutation reported as functionally causative for MH.[23]

Two months after completion of the genetic analysis, the same MH testing center was contacted by a surgeon who was planning an elective surgery on the patient's identical twin brother, which we denote as twin B. Preoperative genetic analysis identified the same Arg2454Cys RYR1 mutation in twin B. Furthermore, twin B also reported a history of 2 episodes of unexplained ER with CKs >18,000 U/L. He also gave a history of 2 episodes of "cardiac arrest" in association with anesthesia as a child while undergoing bilateral ptosis surgery, although no anesthetic records were available for review. He and his family were never told that these episodes might be MH related. Genetic analysis of the parents revealed the same RYR1 mutation in the mother, who also reported a history of muscle cramping. After genetic testing, oral dantrolene (100 mg 3 times per day) was prescribed for each brother for treatment of their debilitating muscle cramping and fatigue. However, while their complaints of muscle cramping, pain, and fatigue were decreased while receiving this dantrolene regimen, neither brother was able to tolerate even moderate exercise.

Both brothers moved to another state and were treated by a primary care provider who discontinued their oral dantrolene due to his unfamiliarity with their disorder and the treatment regimen. Shortly after stopping their dantrolene therapy, the brothers developed recurrent rhabdomyolysis associated with simple routine physical activity. Twin B reported to an ED with a complaint of incapacitating spontaneous muscle pain without exercise. His CK was measured at 2500 U/L, and he received IV hydration and was sent home. When his symptoms continued, he returned to the ED the next day, where his CK was measured at 5000 U/L. He was admitted for IV hydration and given an oral nonsteroidal anti-inflammatory medication and opioids for pain. His pain continued, and by hospital day 2, his CK had increased to 10,000 U/L. He informed the hospitalist in charge of his care that he was MHS and had a RYR1 mutation. The hospitalist was unfamiliar with this condition, and a MH testing center director and Malignant Hyperthermia Association of the United States (MHAUS) hotline consultant were independently contacted for advice.

Both the MH testing center director and MHAUS hotline consultant gave the same advice: admit the patient to the intensive care unit (ICU), monitor hemodynamics, administer IV dantrolene 2.5 mg/kg, and check arterial blood gases and CK every 6 hours. The hospitalist was instructed to retreat with IV dantrolene 1 mg/kg if the CK did not decrease after 6 hours, and to consult the hospital's anesthesiologists and pharmacy for assistance with the dantrolene preparation. Neither the anesthesiologist nor pharmacist was familiar with rhabdomyolysis occurring in an awake MHS patient with RYR1 mutations. Because he was uncomfortable providing treatment, the hospitalist transferred the patient to a larger city hospital with an ICU for higher acuity patients. He did not contact or inform the MH testing center director or hotline consultant of this transfer, nor did the physician at the receiving hospital contact them for advice. After 2 days at the city hospital, the new hospitalist sought advice from the MH testing center director and received the same advice regarding dantrolene therapy. However, this advice was never followed, and the patient was discharged home still in pain with a CK of 3800 U/L after a total of 5 hospital days wherein he received only IV hydration and opioid therapy.

Two weeks later, twin B presented to the hospital ED again, stating that his muscle pain never resolved after the last hospital admission. His CK was 3500 U/L. A new hospitalist contacted the MH testing center director and followed the recommended advice of ICU admission with dantrolene therapy (2.5 mg/kg IV). After 1 dosage of dantrolene and 1 day in the ICU, his CK decreased to 900 U/L. His pain level decreased, and he was discharged home with some residual weakness.

DISCUSSION

Based on a positive CHCT[24] and the presence of a MH-causative RYR1 mutation,[3] twin A was diagnosed as MHS. The patient's mother and twin B were likewise diagnosed as MHS based on the presence of the same mutation. Among the >400 RYR1 variants associated with

MH,[25] Arg2454Cys is one of only 31 functionally characterized MH-causative gene mutations.[23] In this case report, 3 people have been diagnosed as being as MHS, but none is reported to have experienced anesthesia-related MH episodes, including the index case. Although twin B did give a history of cardiac complications in association with general anesthesia as a child, old hospital records were not available for review, and he was never diagnosed with or treated for MH. Furthermore, none of his family members reported a similar history when questioned independently.

Despite advances in molecular genetics, the CHCT remains the only validated diagnostic test for MH and has several limitations. The CHCT was validated by comparing muscle biopsy tissue from patients with documented histories of anesthesia-related MH to muscle biopsy tissue from patients without such a history.[24] Since the MH syndrome is characterized by variable expression and penetrance, it is difficult to know how many subjects in the control population were really MHS. With a specificity of 78%, the CHCT erroneously diagnoses 22% of the population as MHS.[26] However, more problematic is using the CHCT to diagnose people as MHS who present with ER, given that the CHCT was not developed or validated to evaluate patients with ER.

It is important to note that the CHCT was never validated regarding various ethnic groups. Most patients involved in the validation process were Caucasian. The African American population has baseline CK levels higher than those of Caucasians,[27] and these 2 populations have separate and distinct variations in RYR1.[9] Given the differences in baseline CK measurements and RYR1 expression between the African American and Caucasian populations, CHCT results should be interpreted with caution. The African American patient evaluated in this case was diagnosed as MHS based on CHCT thresholds validated in a Caucasian population. However, based on the patient's strong CHCT contractures and Arg2454Cys RYR1 mutation, MHS is a logical diagnosis. The danger with using this methodology potentially arises when a MHS diagnosis is made for an ER patient whose muscle contractures barely reach the positive CHCT criteria, and in whom no MH-causative gene mutation is identified.

Regardless of the impact of ethnicity on RYR1 expression and CHCT thresholds, or the validity of using the CHCT to diagnose patients with ER, it remains undeniable that these twin brothers have a MH-related RYR1 myopathy that responds favorably to oral dantrolene. This is not the typical MH presentation with which most anesthesiologists are familiar, nor is the administration of oral dantrolene a well-recognized therapy for the management of muscle pain in patients who are MHS. Conversely, most ED physicians, hospitalists, and intensivists do not associate ER with MH. Thus, consideration of RYR1 myopathies in the differential diagnosis of ER may be easily overlooked. Although the incidence of fulminant MH during anesthesia is relatively rare, with estimates between 1 in 4200 and 250,000,[28] 2 independent studies estimate the incidence of RYR1 mutations in the general population at 1 in 2000.[29,30] This implies that there is a cohort of MHS patients that does not develop MH during general anesthesia. Furthermore, exome sequencing performed on 870 volunteers without medical or family histories for MHS identified 3 with RYR1 variants predicted to predispose to MH.[31]

Despite the growing clinical evidence describing nonanesthesia-associated MH-related RYR1 myopathies,[4–11,15–21] the lack of human laboratory studies linking MH, ER, and heat stroke has led some investigators to argue against a relationship.[12] However, heat and/or exercise stress triggers nonanesthesia-related MH-like reactions in genetically engineered mice that express RYR1 causal mutations,[32,33] and in some strains of swine with RYR1 mutations.[34] Furthermore, a recent study in humans suggests that RYR1 mutations may account for more than one-third of all unexplained cases of rhabdomyolysis.[11] While no randomized controlled human trials have been conducted to prove that MH, ER, and heat stroke are caused by the same RYR1 mutations, it is unlikely that such a study will ever occur. The incidence of MH or RYR1-related ER and heat stroke will require collaboration among epidemiologists and clinicians expert in sports medicine, neurology, and anesthesiology. Case reports such as this one will hopefully stimulate others to look more deeply into the underlying causes of ER and heat stroke.

Perhaps the most important feature of this case report is the suggestion of a new mission for anesthesiologists to extend their expertise beyond the operating room and share it with primary care providers and ED physicians treating patients with RYR1 myopathies. Likewise, the physicians and scientists associated with MHAUS must broaden their mission and disseminate information to all clinicians regardless of specialty.

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Ligands for FKBP12 Increase Ca^{2+} Influx and Protein Synthesis to Improve Skeletal Muscle Function*

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Background: Rapamycin is a known inhibitor of protein synthesis but also modulates the activity of RyR1.
Results: FKBP12 deficiency and low doses of either rapamycin or SLF increase, rather than decrease, protein synthesis and improve muscle function.
Conclusion: In skeletal muscle, FKBP12 regulates Ca^{2+} influx, Ca^{2+} store refilling, and protein synthesis.
Significance: This study lays the groundwork for the development of interventions to slow muscle fatigue.

Rapamycin at high doses (2–10 mg/kg body weight) inhibits mammalian target of rapamycin complex 1 (mTORC1) and protein synthesis in mice. In contrast, low doses of rapamycin (10 μg/kg) increase mTORC1 activity and protein synthesis in skeletal muscle. Similar changes are found with SLF (synthetic ligand for FKBP12, which does not inhibit mTORC1) and in mice with a skeletal muscle-specific FKBP12 deficiency. These interventions also increase Ca^{2+} influx to enhance refilling of sarcoplasmic reticulum Ca^{2+} stores, slow muscle fatigue, and increase running endurance without negatively impacting cardiac function. FKBP12 deficiency or longer treatments with low dose rapamycin or SLF increase the percentage of type I fibers, further adding to fatigue resistance. We demonstrate that FKBP12 and its ligands impact multiple aspects of muscle function.

The macrolide rapamycin is a potent and widely used immunosuppressant that, at doses from 2 to 10 mg/kg has many beneficial physiological effects in mice (1), including increased life span (2); improved cognitive function (3); and alleviation of symptoms of cancer (4, 5), tuberous sclerosis complex (6), Alzheimer disease (3), Leigh syndrome (7), and muscle disease (8). Most of these effects are ascribed to the inhibition of mammalian target of rapamycin complex 1 (mTORC1)[7] (9, 10). To effect this mechanism, rapamycin acts as a pro-drug that binds to FKBP12 (FK506-binding protein 12 kDa) creating a composite surface that binds to the protein kinase mTOR, blocking phosphorylation of mTORC1 targets. This decreases protein synthesis and promotes autophagy. However, FKBP12 is a peptidylprolyl isomerase and a binding partner for several important signaling proteins (11–13). FKBP12 ligands, including rapamycin, FK-506, ascomycin, and the synthetic molecule SLF (synthetic ligand for FKBP12), can disrupt these interactions (14, 15).

FKBP12 binding to the skeletal muscle Ca^{2+} release channel RyR1 stabilizes a closed state of the channel to minimize Ca^{2+} leak from the sarcoplasmic reticulum (SR) (11). FKBP12 also binds to TGFβR1 and slows phosphorylation by TGFβR2 to decrease TGFβ signaling at low ligand concentrations (12) and binds to palmitoylated Ras to promote depalmitoylation and movement from the plasma membrane to the endoplasmic reticulum/Golgi (13). At present, there is little information regarding alternative protein targets of FKBP12 and their role in the physiological effects of rapamycin in skeletal muscle.

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[7] The abbreviations used are: mTORC1, mammalian target of rapamycin complex 1; mTOR, mammalian target of rapamycin; FKBP12D, mouse heterozygous for floxed FKBP12 gene and heterozygous for FKBP12 knockout (lox/null) expressing CRE recombinase under the muscle creatine kinase promoter; RyR1, sarcoplasmic reticulum Ca^{2+} release channel in skeletal muscle or ryanodine receptor; RyR3KO, mice with a general knockout of RYR3 generated by H. Takeshima; SR, sarcoplasmic reticulum; $Ca_V1.1$, skeletal L-type voltage-dependent calcium channel; FDB, flexor digitorum brevis; EDL, extensor digitorum longus; SERCA, SR Ca^{2+}-ATPase; CSQ, calsequestrin; ERR, estrogen-related receptor; PPAR, peroxisome proliferator-activated receptor; SLF, synthetic ligand for FKBP12; BTS, 4-methyl-*N*-(phenylmethyl)benzenesulfonamide.

Excitation-contraction coupling in skeletal muscle culminates in the release of Ca^{2+} from the SR to the cytoplasm, leading to force development. For the muscle to contract, the depolarization of the transverse tubule membrane in response to a signal from the nerve induces a conformational change in the voltage-dependent Ca^{2+} channel, $Ca_V1.1$, which, in turn, mechanically gates the opening of RyR1, releasing Ca^{2+} from SR Ca^{2+} stores. Muscle relaxation requires a decrease in the cytoplasmic Ca^{2+} levels, the majority of which is pumped back into the store via SR Ca^{2+}-ATPase (SERCA).

With repetitive activation, skeletal muscle exhibits a decline in force generation, a process designated as fatigue. The rate at which muscle fatigues is inversely related to the proportion of oxidative, slow twitch, type I fibers, which fatigue more slowly than type II fibers (16). Skeletal muscle fiber type composition displays some plasticity and can be modulated by exercise (17), due to the activation of the calcineurin/NFAT (18) and estrogen-related receptor γ (ERRγ)/PPARβ/γ pathways (19). Interventions that activate these pathways also increase the proportion of type I fibers and slow muscle fatigue (20). The maintenance of SR Ca^{2+} stores and the magnitude of the Ca^{2+} transients are also critical factors in slowing muscle fatigue (18–21). *In vitro* repetitive contractions reduce SR Ca^{2+} stores and the amount of SR Ca^{2+} that is released, leading to skeletal muscle fatigue (21).

Given the multisystemic effects of rapamycin and FKBP12 modulation, it is important to determine whether alterations in FKBP12 and rapamycin targets other than mTORC1 contribute to the effects of this drug. In this paper, we explore the effects of genetic and pharmacological disruption of FKBP12 on Ca^{2+} signaling, protein synthesis, and function of skeletal muscle. We have evaluated the effects of low dose (10 μg/kg body weight) rapamycin and SLF (1 μg/kg body weight) on skeletal muscle function and compare the drug effects with a skeletal muscle-specific deficiency in FKBP12. SLF does not alter mTORC1 or calcineurin activity (22). We demonstrate that rapamycin, SLF, and muscle-specific deficiency in FKBP12 enhance the refilling of SR Ca^{2+} stores, turn on the slow fiber-type program, slow muscle fatigue, and improve running endurance in mice. We also show that these treatments increase, rather than decrease, protein synthesis. In our studies, the first detectable effect of both rapamycin and SLF is an alteration in Ca^{2+} influx to enhance refilling of Ca^{2+} stores. We propose that this is the primary mechanism whereby low doses of these drugs improve endurance. However, other targets may be involved in some of the other effects of low dose rapamycin and SLF.

MATERIALS AND METHODS

Drugs—Rapamycin was purchased from LC Laboratories, and SLF was purchased from Cayman Chemical Company.

Antibodies—Antibodies for phospho-S6 (Ser-235/236), S6, phospho-4-EBP1 (Thr-37/46), 4EBP1, phospho-Akt (Ser-473), phospho-Akt (Thr-308), Atg5, beclin 1, phospho-SMAD2 (Ser-465/467), SMAD2, phospho-eEF2 (Thr-56), and eEF2 were obtained from Cell Signaling. Antibodies for SERCA1, SERCA2, calsequestrin, RyR1, FKBP12, and $Ca_V1.1$ were obtained from Thermo Scientific. Antibodies for myoglobin, Akt, phospho-ERK (Thr-202/Tyr-204), and ERRα were obtained from Santa Cruz Biotechnology, Inc. Antibodies to MHC1 and MHC2a were from DSHB. The antibody to puromycin was obtained from Kerafast.

Mouse Models—The FKBP12D mice (skeletal muscle-specific FKBP12-deficient mice) were created in our laboratory as described by Tang *et al.* (23). Mice (CRE^+) expressing CRE^+ recombinase under the creatine kinase promoter were used as controls. The RyR3 knock-out mice were generously provided by Dr. H. Takeshima. Mice used were between 6 and 12 weeks of age except where indicated and were age-matched in each experiment. All mice were housed at room temperature with a 12-h/12-h light/dark cycle. Food and water were provided *ab libitum*. All procedures were approved by the Animal Care Committee at Baylor College of Medicine.

Echocardiography—Echocardiography was performed using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) with a 30-MHz probe. Mice were initially anesthetized with 2% isoflurane mixed with 100% O_2 and placed on a heated pad, where all four limbs were taped down onto copper electrocardiogram plates. On the plate, anesthesia was maintained with 1% isoflurane through a nose cone. A rectal temperature probe was used to monitor body temperature.

MHC Fiber Typing—Soleus, EDL, diaphragm, and flexor digitorum brevis (FDB) muscles were dissected, embedded (Tissue-Tek), and frozen in the 2-methylbutane (Sigma) pre-cooled in the liquid nitrogen. The frozen muscles were sectioned at 10-μm thickness using a cryostat microtome (SHANDON Cryotome E, Thermo Electron Corp.). Sections were stained with myosin heavy chain-specific antibodies and isotype-specific secondary antibodies and imaged under the fluorescence microscope (Olympus). The relative numbers of the different fiber types were quantified and normalized by the total number of muscle fibers per field of images.

Calcineurin Activity Assay—Calcineurin activity was evaluated with a colorimetric assay kit from Calbiochem/Millipore. Diaphragm, soleus, and EDL muscle were homogenized according to the kit instructions and were desalted before the assay. Samples were incubated at 37 °C for 30 min. The activity was normalized to total protein in the sample homogenates.

SR Membrane and Homogenate Preparation—Homogenates from diaphragm, EDL, and soleus muscles were prepared in buffer containing 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 10 mM Tris-HCl, pH 6.8, and protease inhibitors as in the membrane preparations. Muscles were homogenized in the Precellys 24 tissue homogenizer and centrifuged in an Eppendorf microcentrifuge at 25,000 × *g* for 15 min. Supernatants were aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Protein concentration was measured by a Lowry and/or BCA assay.

Calculation of Percentage Occupancy of FKBP12 Sites on RyR1—Intracellular FKBP12 concentration was calculated using μmol of FKBP12/mg of protein in an aliquot of the homogenate, the amount of total protein, and volume of the muscle bundles. The volume was estimated from the mass and the density of 1.06 mg/ml. To approximate the "free concentration" of FKBP12, homogenates were separated into supernatants and pellets by centrifugation in a Beckman Airfuge under conditions where membranes, nuclei, and mitochondria are found in

the pellet and soluble proteins are found in the supernatant. To control for homogenization efficiency, we assessed the distribution of myoglobin in the supernatants and pellets using an anti-myoglobin antibody. The total amount of FKBP12 in each supernatant and pellet was calculated and corrected for the solubilization efficiency. To estimate the concentration of FKBP12 binding sites associated with RyR1, we multiplied the number of [^{3}H]ryanodine binding sites by 4 (one binding site per subunit of the homotetramer). The total RyR1 in muscle homogenates of the soleus, EDL, and diaphragm muscle in FKBP12D and CRE$^+$ mice using RyR (nM) was estimated from the expression, 1000 dBP/m, where d is the density of 1.06 mg/ml, B is the amount of RyR1 in the muscle estimated from [^{3}H]ryanodine binding in nmol/mg, P is the total protein measured in homogenates, and m is the mass of the muscle. We also determined the affinity of [^{35}S]FKBP12 for muscle membranes at room temperature ($K_D = 8.6 \pm 1.2$ nM, $n = 7$) and at 37 °C ($K_D = 27 \pm 2.2$ nM, $n = 3$). The estimated number of FKBP12s bound per RyR1 was determined in the soleus, EDL, and diaphragms of CRE$^+$ and FKBP12D mice. We estimate that in CRE$^+$ muscle, there is adequate FKBP12 to saturate >95% of the RyR1 binding sites, and despite a 80–90% decrease in tissue FKBP12 in the FKBP12D muscle, the saturation of RyR1 would decrease by <50% in most muscles.

Endurance Running—Endurance running was performed as described by Narkar *et al.* (20) with modifications.

Grip Strength—FKBP12D and CRE$^+$ mice (7.5–8.5 weeks old) were tested for grip performance on a grip strength meter from Columbus Instruments, according to the standard protocol from Jackson Laboratory. For each mouse, the test was repeated five consecutive times for both hind limb and forelimb together.

Fatigue—Fatigue studies were performed as described previously (24).

Manganese Quench Assay—Isolated FDB muscle fibers were loaded with 10 μM Fura-2/AM for 1 h at room temperature in DMEM in the presence of 20 μM BTS. The fluorescent signal of preloaded fibers was monitored with a calcium-insensitive excitation filter, 360 nm, placed in a Lambda DG4 system used as light source. Emitted florescence was collected through a Nikon S. Fluor objective (×20, 0.75 numerical aperture) coupled to an inverted microscope (Nikon eclipse TE-200) and digitized using a CCD Rolera MGi-Plus camera with 510 × 252 pixels field size (using 6 binning). Data were collected and stored in the computer using the Metafluor software (version 6.2) for further analysis. The fluorescent signal was measured during 5 min in the presence of normal Tyrode buffer, and then $CaCl_2$ was replaced with $MnCl_2$ (1.8 mM) for the rest of the experiment; at min 10, the focused FDB was stimulated with 20 trains. Electrical stimulation was performed using two platinum wires placed at each side of the fiber oriented longitudinally, and one electrical train (50 Hz, 1 s duration) was applied every 5 s (20 trains total).

Ca^{2+} Measurements—Note that myofibers were loaded with the Ca^{2+} indicators at room temperature because we found that 37 °C loading caused the dyes to partition into various organelles and gave results very different from those we are currently reporting. To monitor the Ca^{2+} release kinetics with electrical stimulation, FDB fibers were loaded with 5 μM Mag-Fluo4 for 30 min at room temperature in the presence of 20 μM BTS. Loaded FDBs were perfused with Tyrode buffer with 20 μM BTS at 0.5 ml/min) and imaged in line scan mode (one line was acquired every 1.15 ms (3.66 μs/pixel time) using a ×20 objective (EC Plan-Neofluar) mounted in the confocal microscope (Zeiss LSM 510 meta)). Dye was excited at 488 nm, and emitted light was collected through an LP 505-nm filter.

Western Blots—Western blots were performed as described previously (24). To control for variations among different Western blots and allow for multiple repeats, the absorbance intensity for each Western blotted band was normalized to the absorbance intensity of the corresponding band from the control mice (WT, CRE$^+$, or WT saline) from the same Western blot, averaged with values obtained from other Western blots, and plotted as a percentage of control. Some blots had multiple controls, and the absorbance of the specific band was normalized to the average.

Detection of Puromycin-labeled Proteins—Mice (6–8 weeks of age) were food-deprived for 6 h and refed for 2 h.

Propofol (18 μl/g) was injected (intraperitoneally) in mice after 70 min of refeeding. The mice were then injected (intraperitoneally) with puromycin (0.04 μmol/g body weight), and the mice were sacrificed 35 min later. Muscles were isolated, homogenized, and prepared for Western blotting with antipuromycin antibody. For normalization to total protein, the same Western blots were stained with the Swift Membrane Stain™ kit (G-Biosciences).

Statistics—Data are presented as mean ± S.E. Statistical analyses of two groups were performed as with Student's t test. $p < 0.05$ was considered statistically significant: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

The Effects of FKBP12 Deficiency, Rapamycin, and SLF on Muscle Function—To determine whether reducing the interaction of FKBP12 with its targets in skeletal muscle contributes to the functional effects of rapamycin, we used three approaches to decrease the FKBP12 interactions without directly inhibiting mTORC1: 1) decreased skeletal muscle FKBP12 expression, 2) low doses of rapamycin, and 3) a drug (SLF) that displaces FKBP12 from RyR1 and other targets without inhibiting mTORC1 or calcineurin.

We created mice with a general knockout of FKBP12 (25), but the homozygous mice (FKBP12$^{null/null}$) died at birth from cardiac failure and/or respiratory insufficiency. Heterozygous FKBP12$^{WT/null}$ mice thrived and closely resembled the wild type (WT) and CRE$^+$ mice in our assays. We also created two strains of mice with deficiencies in FKBP12 in skeletal muscle (23). One strain of mice was homozygous for the floxed allele (FKBP12$^{lox/lox}$), and the second was heterozygous for the floxed allele and null for the second FKBP12 allele (FKBP12$^{lox/null}$, obtained by crossing FKBP12$^{lox/lox}$ with FKBP12$^{WT/null}$ mice). Both types of mice (FKBP12$^{lox/lox}$ and FKBP12$^{lox/null}$) expressed CRE$^+$ recombinase under the creatine kinase promoter and are on a C57BL6/J background. The magnitude of the muscle decrease in FKBP12 is shown in Fig. 1*A*. FKBP12 was decreased by 80–90% in most muscles of both types of FKBP12

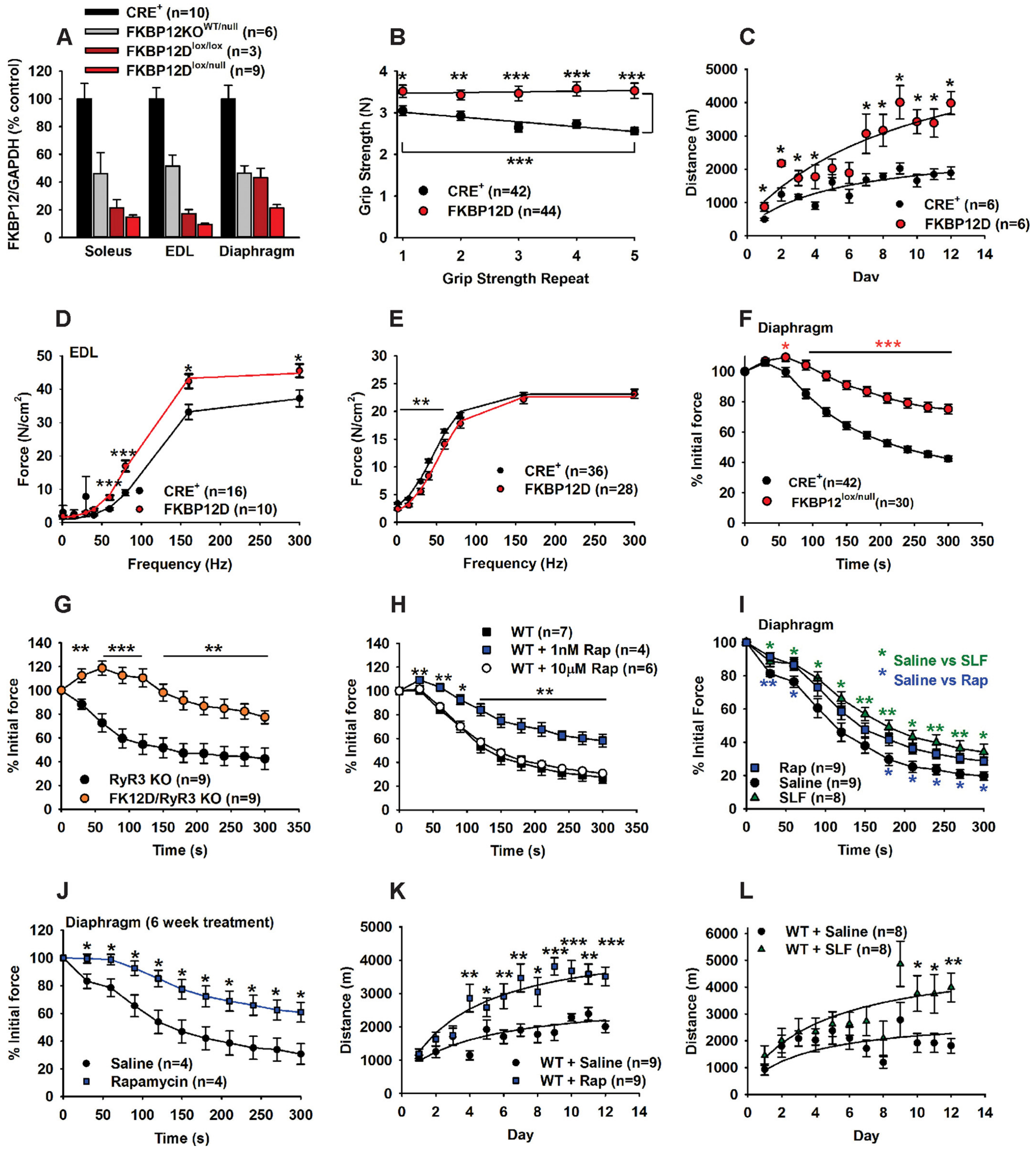

FIGURE 1. **Phenotype of mice with skeletal muscle FKBP12 deficiency.** *A*, levels of FKBP12 in muscle of CRE$^+$, FKBPP12$^{WT/null}$, FKBP12D$^{lox/lox}$, and FKBP12$^{lox/null}$ mice. *B*, grip strength of the four mouse paws, measured in five consecutive repeats. *C*, endurance running. CRE$^+$ and FKBP12D mice were run to exhaustion repeatedly for 12 consecutive days, and total distance run per day was determined. *D*, force-frequency curve for the EDL of CRE$^+$ and FKBP12D mice. *E*, force-frequency curve for the diaphragm muscle of CRE$^+$ and FKBP12D mice. *F*, diaphragm fatigue of CRE$^+$ and FKBP12D mice. *G*, diaphragm fatigue in mice that do not express RyR3 with and without FKBP12 deficiency. *H*, effect of rapamycin treatment on diaphragm fatigue *in vitro*. WT mice were sacrificed, and diaphragms were isolated. 1 nM or 10 μM rapamycin was added, and after 20 min, the force frequency and fatigue studies were performed. *I*, diaphragm fatigue in mice treated for 2 weeks with low dose rapamycin or SLF. *J*, diaphragm fatigue in mice treated for 6 weeks with low dose rapamycin. *K*, endurance running of mice treated with rapamycin. WT mice were injected with saline or rapamycin (10 μg/kg body weight), every other day, 1 h prior to test and run to exhaustion repeatedly for 12 consecutive days. Distance run each day is shown. *L*, endurance running of mice treated with SLF. WT mice were injected with saline or SLF (1 μg/kg body weight), every other day, 1 h prior to test and run to exhaustion repeatedly for 12 consecutive days. Distance run each day is shown. Data are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

knockdown mice except in the diaphragms of the FKBP12$^{lox/lox}$ mice, where the decrease was less than in other muscles and less than in the diaphragms of the FKBP12$^{lox/null}$ mice. For the remainder of this paper, we present the data obtained with the FKBP12$^{lox/null}$ mice because the extent of knockdown of FKBP12 was greater across all muscle groups, the functional consequences of FKBP12 deficiency for muscle function were greater, and the FKBP12 levels varied less in the diaphragm among the FKBP12-deficient mice. For simplicity, these FKBP12$^{lox/null}$ mice are designated FKBP12D.

When compared with the CRE$^+$ mice, the FKBP12D mice had improved grip strength, which did not decline with repetitive trials (Fig. 1*B*) and increased running endurance (Fig. 1*C*). We have previously reported that these mice also display improved muscle recovery from injury (26). Overall, our data suggest that muscle function is greatly improved by FKBP12 deficiency. The data in Fig. 1, *B* and *C*, suggest that fatigue may be less in the FKBP12D mice.

Previous studies with FKBP12D on a mixed background showed small changes in the force-frequency curves (23) that were different from those obtained with muscles from FKBP12D mice back-crossed onto a clean C57B6/J background. With the mice on the C57B6J background, we found no differences in the force frequency curves in the soleus (data not shown), but the EDL of FKBP12D mice generated more force than the corresponding EDL muscles of CRE$^+$ mice, and the half-maximal stimulation frequency was shifted to the left by 17 Hz ($p < 0.001$) (Fig. 1*D*). The force-frequency curve for the diaphragm of FKBP12D displayed a 12-Hz rightward shift ($p = 0.001$) (Fig. 1*E*).

Although we found small but significant slowing of fatigue in the EDL and soleus muscle (data not shown), the most significant effects of FKBP12 deficiency on fatigue were found in the diaphragm (Fig. 1*F*). This slowing of diaphragm fatigue by FKBP12 deficiency was not dependent on the presence of a second isoform of RyR known to be expressed in diaphragm, RyR3, because the slowed fatigue was detected in diaphragms of mice that were deficient in both FKBP12 and RyR3 (Fig. 1*G*).

The finding that FKBP12 deficiency slows muscle fatigue raises the question of whether rapamycin and SLF can also slow fatigue. We tested the effects of rapamycin at two different concentrations on muscle fatigue in WT diaphragms. Treatment of isolated WT diaphragms with 1 nM rapamycin slowed diaphragm fatigue (Fig. 1*H*). In contrast, a high dose of rapamycin (10 μM, a concentration that should remove most of the FKBP12 from RyR1) did not slow diaphragm fatigue *in vitro* (Fig. 1*H*), suggesting that some drug-free FKBP12 is required to slow fatigue.

We also tested the effects of *in vivo* treatment of mice with low dose rapamycin and SLF. WT mice were injected (intreperitoneally) with rapamycin (10 μg/kg) or SLF (1 μg/kg) every other day for 2 weeks. The rapamycin dose used in this study was 50–1000-fold less than that used in mouse studies to inhibit mTORC1 signaling or promote longevity (0.5–10 mg/kg) (27–30). We found that diaphragm fatigue was also slowed by *in vivo* treatment with rapamycin and SLF (Fig. 1*I*). We also tested rapamycin (10 μg/kg every second day) for 6 weeks and found that again diaphragm fatigue was slowed (Fig. 1*J*).

The ability of low dose rapamycin to slow muscle fatigue led us to test *in vivo* low dose rapamycin on endurance running of the mice. We injected (intraperitoneally) mice with rapamycin (10 μg/kg) or SLF (1 μg/kg) every other day for 2 weeks. Consistent with the data obtained with FKBP12D mice, both rapamycin (Fig. 1*K*) and SLF (Fig. 1*L*) improved endurance running. The difference between rapamycin- and saline-treated mice was significant after only two treatments (day 4) and continued to improve during the 12 days of treatment (Fig. 1*K*).

A concern in the use of rapamycin to slow skeletal muscle fatigue is that, even at low doses, rapamycin could negatively impact cardiac muscle function. We found, using echocardiography, that cardiac function was not altered by rapamycin in mice treated for up to 4 weeks (Fig. 2).

The Effects of FKBP12 Deficiency, Rapamycin, and SLF on Fiber Type Distribution—Slowing of muscle fatigue is frequently associated with increases in oxidative fibers. Using myosin heavy chain (MHC) immunohistochemical staining, we found that FKBP12 deficiency and, to a lesser extent, rapamycin and SLF (2-week treatment) activated the slow fiber program to increase oxidative fibers in the diaphragm (Fig. 3, *A*, *D*, and *G*), soleus (Fig. 3, *B*, *E*, and *H*), and EDL (Fig. 3, *C*, *F*, and *I*) compared with control mice.

Effects of Rapamycin, SLF, and FKBP12 Deficiency on Muscle Ca^{2+} Handling—The slowing of muscle fatigue driven by FKBP12 deficiency or low dose rapamycin/SLF could be secondary to changes in intracellular Ca^{2+} handling. FKBP12 regulates the activity of the Ca^{2+} release channel, RyR1, by stabilizing the closed state of the channel, and drugs that completely remove FKBP12 from RyR1 increase SR Ca^{2+} leak (31). To evaluate effects of FKBP12 deficiency and low dose rapamycin and SLF on intracellular Ca^{2+} handling, we used either Fura 2 or Mag-Fluo 4 (loaded into fibers at room temperature; see "Ca^{2+} Measurements") to assess changes in cytosolic Ca^{2+} concentrations, Ca^{2+} transients, and the rate of return of Ca^{2+} poststimulation to baseline. In a single twitch (1 Hz), the rate of return of cytosolic Ca^{2+} to baseline was slower in FKBP12D fibers than in CRE$^+$ fibers (Fig. 4*A*). This could have reflected slowed reuptake of Ca^{2+} into the SR, increased Ca^{2+} influx, or decreased cytosolic Ca^{2+} buffering. With repetitive 50-Hz trains (representative transients shown in Fig. 4*B*), both the poststimulation cytosolic Ca^{2+} (Fig. 4*C*) and the peak amplitude of the Ca^{2+} transient (Fig. 4*D*) increased in FKBP12D compared with CRE$^+$ fibers, suggesting enhanced rather than decreased refilling of SR Ca^{2+} stores and a possible net gain of Ca^{2+} to the fiber. Although Ca^{2+} enters the fiber during stimulation primarily through the voltage-gated Ca^{2+} channel, $Ca_V1.1$, this channel shuts down immediately following stimulation.

In response to repetitive tetanic (100-Hz) stimulation, the Ca^{2+} transient amplitude declined more slowly in FKBP12D compared with CRE$^+$ fibers (Fig. 4*E*). Similar results were obtained with 10 nM rapamycin and 1 nM SLF in fibers from WT mice (Fig. 4, *F* and *G*). Poststimulation 4-chloro-*m*-cresol (4CmC) releasable Ca^{2+} stores (Fig. 4*H*) were higher in fibers from FKBP12D compared with CRE$^+$ mice and in SLF- and

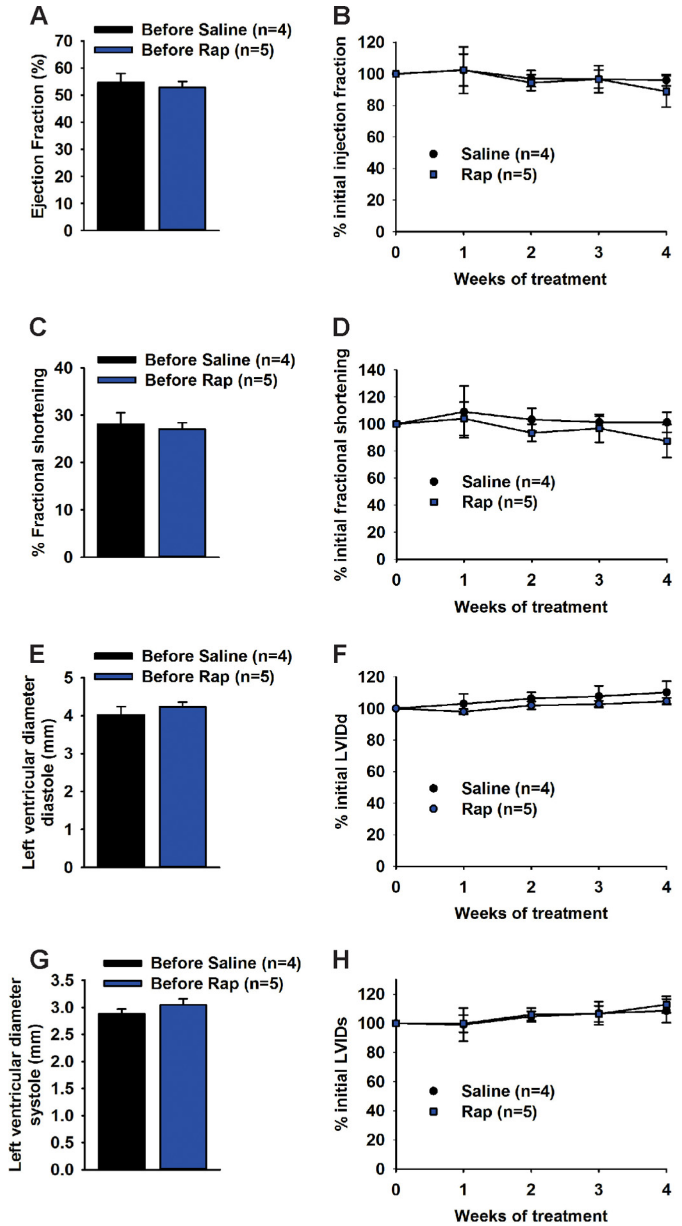

FIGURE 2. **Effects of low dose rapamycin on cardiac function.** 6–8-week-old C57/Bl6J mice were treated for 4 weeks with rapamycin (10 μg/kg), and cardiac function was measured using echocardiography once per week. *A*, average ejection fraction before rapamycin treatment. *B*, ejection fraction over time as a percentage of the initial measurement. *C*, average fractional shortening before rapamycin treatment. *D*, fractional shortening over time as a percentage of the initial measurement. *E*, average left ventricular internal diameter at diastole before rapamycin treatment. *F*, left ventricular internal diameter at diastole over time as a percentage of the initial measurement. *G*, average left ventricular internal diameter at systole before rapamycin treatment. *H*, left ventricular internal diameter at systole over time as a percentage of the initial measurement. Data are shown as mean ± S.E. (*error bars*).

rapamycin-treated compared with untreated fibers from WT mice. Increased SR Ca^{2+} stores and diminished decline of Ca^{2+} transients with repetitive fatiguing stimulation again suggested a net increase of Ca^{2+} in the fibers. To explore this possibility, we used a manganese quench assay to assess changes in Ca^{2+} influx into the fiber. Electrically stimulated Ca^{2+} influx into FDB fibers was enhanced by SLF, rapamycin, and FKBP12 deficiency (*manganese quench curves* in Fig. 4, *I–K*, and *slopes* in Fig. 4*L*).

These data suggest that the drugs and FKBP12 deficiency are enhancing the opening of a Ca^{2+} influx pathway during electrical stimulation. The most obvious possibility is $Ca_V1.1$, the voltage-dependent Ca^{2+} channel that mechanically gates RyR1 opening, but channels activated subsequent to excitation-contraction coupling may also contribute. Both enhanced refilling of SR Ca^{2+} stores and increased oxidative fibers are likely to underlie the improved endurance of the FKBP12-deficient and the SLF/rapamycin-treated mice. In addition, changes in the amplitude of the Ca^{2+} transient and the poststimulation cytosolic Ca^{2+} concentrations impact other pathways that respond to alterations in the amplitude, duration, and location of Ca^{2+} signals.

Estimation of the Effects of FKBP12 Deficiency on the FKBP12-RyR1 Interaction—Although FKBP12 has other targets in skeletal muscle (*e.g.* TGFβR1, Ras), little is known about the affinity of these proteins for FKBP12. The most abundant FKBP12-binding protein in skeletal muscle is RyR1. Complete removal of FKBP12 from RyR1 induces SR Ca^{2+} leak (32); however, no assessment of effects on intracellular Ca^{2+} regulation of partial removal of FKBP12 from RyR1 has been performed. We estimated the effects of FKBP12 deficiency on the FKBP12-RyR1 interaction (see complete description of the approach under "Materials and Methods"). Using Western blotting and purified FKBP12 to generate a standard curve (Fig. 5, *A* and *B*), we calculated the total concentration of FKBP12 in the soleus, EDL, and diaphragms of CRE^+ mice to be 1.6 ± 0.3, 1.3 ± 0.2, and 3.0 ± 0.5 μM (n = 7), respectively. In FKBP12D mice, the total FKBP12 concentrations in the soleus, EDL, and diaphragm were calculated to be 0.1 ± 0.06, 0.08 ± 0.02, and 0.37 ± 0.07 μM (n = 7), respectively. The concentration of FKBP12 binding sites on RyR1 (four FKBP12 binding sites per RyR1 tetramer) was between 200 and 400 nM, and the affinity of FKBP12 for RyR1 was 8–30 nM, depending on temperature (Fig. 5, *C* and *D*). There is adequate FKBP12 in CRE^+ muscle to saturate >95% of the binding sites on RyR1, and, despite a 80–90% decrease in FKBP12 levels in the FKBP12D muscle, there is adequate FKBP12 in FKBP12D muscle to decrease its interaction with RyR1 by less than 50% in most muscles. RyR1 protein levels tended to decrease in FKBP12 diaphragm (Fig. 5, *E* and *F*), but RyR1 mRNA levels were unchanged (Fig. 5*H*). $Ca_V1.1$ protein levels (Fig. 5, *E* and *G*) and mRNA (Fig. 5*I*) were not altered by FKBP12 deficiency. Neither RyR1 oxidation nor phospho-Ser-2844 phosphorylation is altered by FKBP12 deficiency (data not shown).

Other Ca^{2+}-handling Proteins—Changes in SR Ca^{2+} stores could arise from secondary changes in the proteins that regulate SR Ca^{2+} release, SR Ca^{2+} uptake, or SR Ca^{2+} buffering. FKBP12.6, another immunophilin that binds and modulates RyR1 was present in skeletal muscle at levels below the detection with our antibodies, but the mRNA levels for FKBP12.6 were not altered in the FKBP12D muscle (Fig. 6*A*). Western blots for the sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1), SERCA2, and calsequestrin (CSQ) are shown in Fig. 6*B*. Consistent with the increase in percentage of type I fibers, SERCA1 (found in type II fibers) protein levels were decreased in the muscle FKBP12D mice (Fig. 6*C*). SERCA2 protein levels were increased in the soleus and the diaphragm of the FKBP12 mice (Fig. 6*D*). Calsequestrin 1 and 2

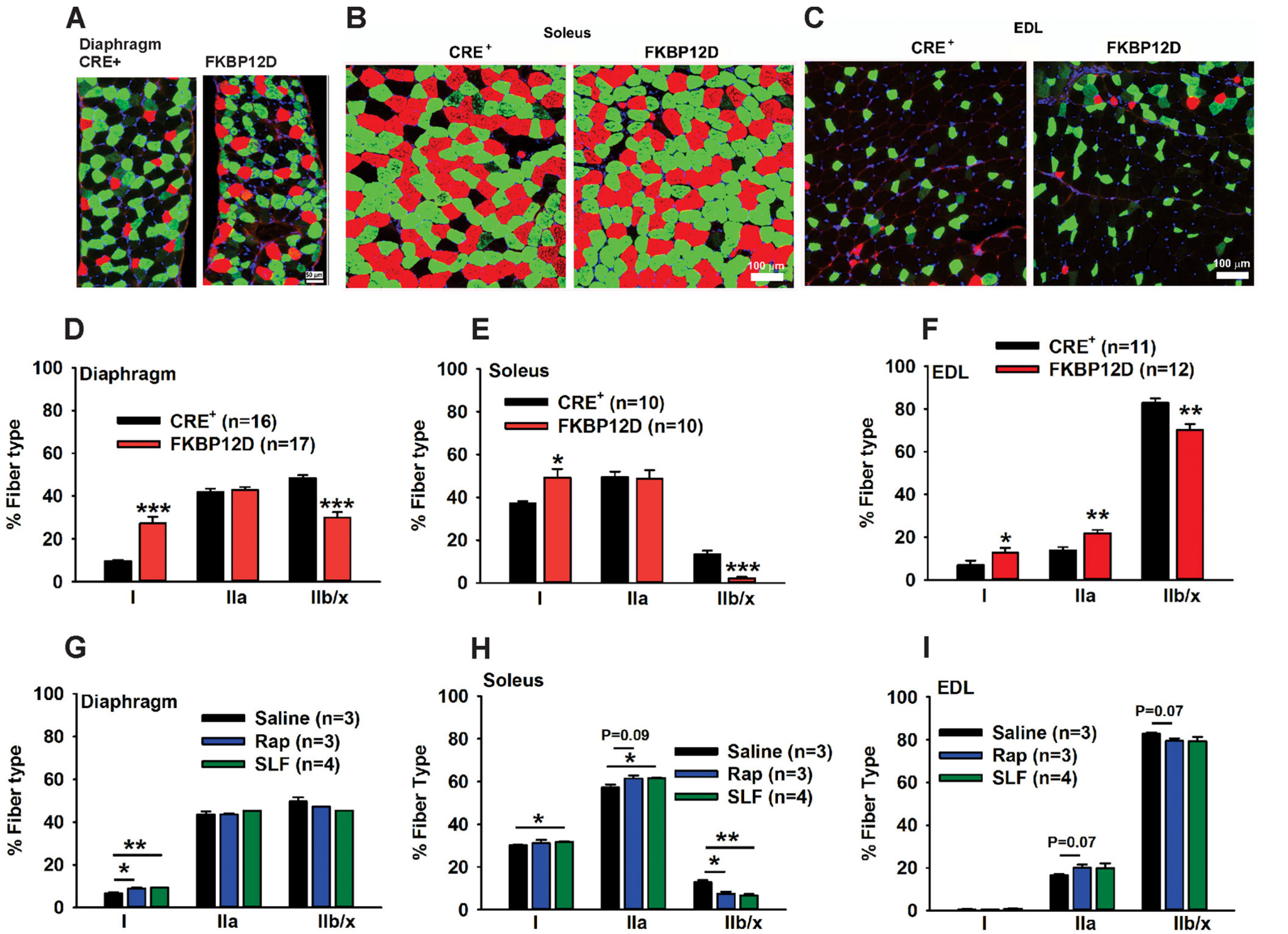

FIGURE 3. **Effect of FKBP12 deficiency and low dose rapamycin and SLF on fiber type distribution.** *A*, MHC fiber type staining in CRE$^+$ and FKBP12D diaphragms by MHC immunocytochemistry. Type I fibers are *pseudocolored red*. Type IIa fibers are *pseudocolored green*, and type IIb/x fibers are *black*. Mice were 7–9 weeks old. *B*, MHC fiber type staining in CRE$^+$ and FKBP12D soleus by MHC immunocytochemistry. *C*, MHC fiber type staining in CRE$^+$ and FKBP12D EDL by MHC immunocytochemistry. *D*, fiber type distribution in the diaphragm of FKBP12D and CRE$^+$ mice. *E*, fiber type distribution in the soleus of FKBP12D and CRE$^+$ mice. *F*, fiber type distribution in the EDL of FKBP12D and CRE$^+$ mice. *G*, analysis of diaphragm fiber type changes after low dose rapamycin or SLF. WT mice were injected with saline, rapamycin (10 μg/kg body weight), or SLF (1 μg/kg body weight) every other day. At the end of 2 weeks, mice were sacrificed, and muscles were isolated and sectioned with a cryostat for staining with MHC antibodies. Mice were 8–11 weeks of age. *H*, analysis of soleus fiber type changes after low dose rapamycin or SLF. *I*, analysis of EDL fiber type changes after low dose rapamycin or SLF. Data in the figure are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(CSQ1/2), which binds Ca^{2+} in the luminal SR, is increased in the soleus of FKBP12D mice, but CSQ1/2 levels were not statistically different in the EDL or diaphragm (Fig. 6*E*).

FKBP12 Deficiency and Rapamycin Increase Protein Synthesis—Given that rapamycin is a known inhibitor of mTORC1 and, hence, protein synthesis in mice at doses between 2 and 10 mg/kg, we assessed the effects of low dose (10 μg/kg) rapamycin, SLF (1 μg/kg), and FKBP12 deficiency on protein synthesis using a modified SUnSET assay (33). To minimize variations in protein synthesis associated with variations in time of food intake, we deprived mice (CRE$^+$, FKBP12D, and WT) of food for 6 h, followed by a single intraperitoneal injection with saline, rapamycin, or SLF, and then food was provided for 2 h prior to sacrifice. The mice were injected with puromycin 35 min before the sacrifice. FKBP12 deficiency (Fig. 7, *A* and *B*) and low dose rapamycin and SLF treatment (Fig. 7, *C* and *D*) increased protein synthesis.

An increase, rather than a decrease, in protein synthesis suggests that at a low dose, rapamycin is activating rather than inhibiting mTORC1. We assessed the effects of FKBP12 deficiency, low dose rapamycin, and SLF on signal transduction pathways both upstream (phospho-Akt (Ser-473)/Akt and phospho-Akt (Thr-308)/Akt) and downstream of mTORC1 (phospho-S6 (Ser-235/Ser236), T46-4EBP14E-BP1 (Thr-37/Thr-46)/4E-BP1, and phospho-eEF2 (Thr-56)/eEF2) that modulate the rate of protein synthesis. We also assessed the status of MAPK signaling by analyzing phospho-ERK/ERK and ribosome levels (also regulated by mTORC1) by examining the levels of L7a normalized to GAPDH. Note that all data are normalized to the Western blot values for the WT or CRE$^+$ controls from the same Western blot, which allowed us to do multiple replicates (each normalized to their own controls) to ensure significance. For the drug treatments, mice were given a single dose of either rapamycin or SLF, and signal transduction changes were assessed after 2 h. Mice were sacrificed 2 h after administration of the drugs.

Mice (either FKBP12D (Fig. 8, *A–D*) or treated for 2 h (Fig. 8, *E–H*) or 2 weeks (Fig. 8, *I–L*) with low dose rapamycin or SLF) displayed pronounced increases in both phospho-S6 (Ser-235/

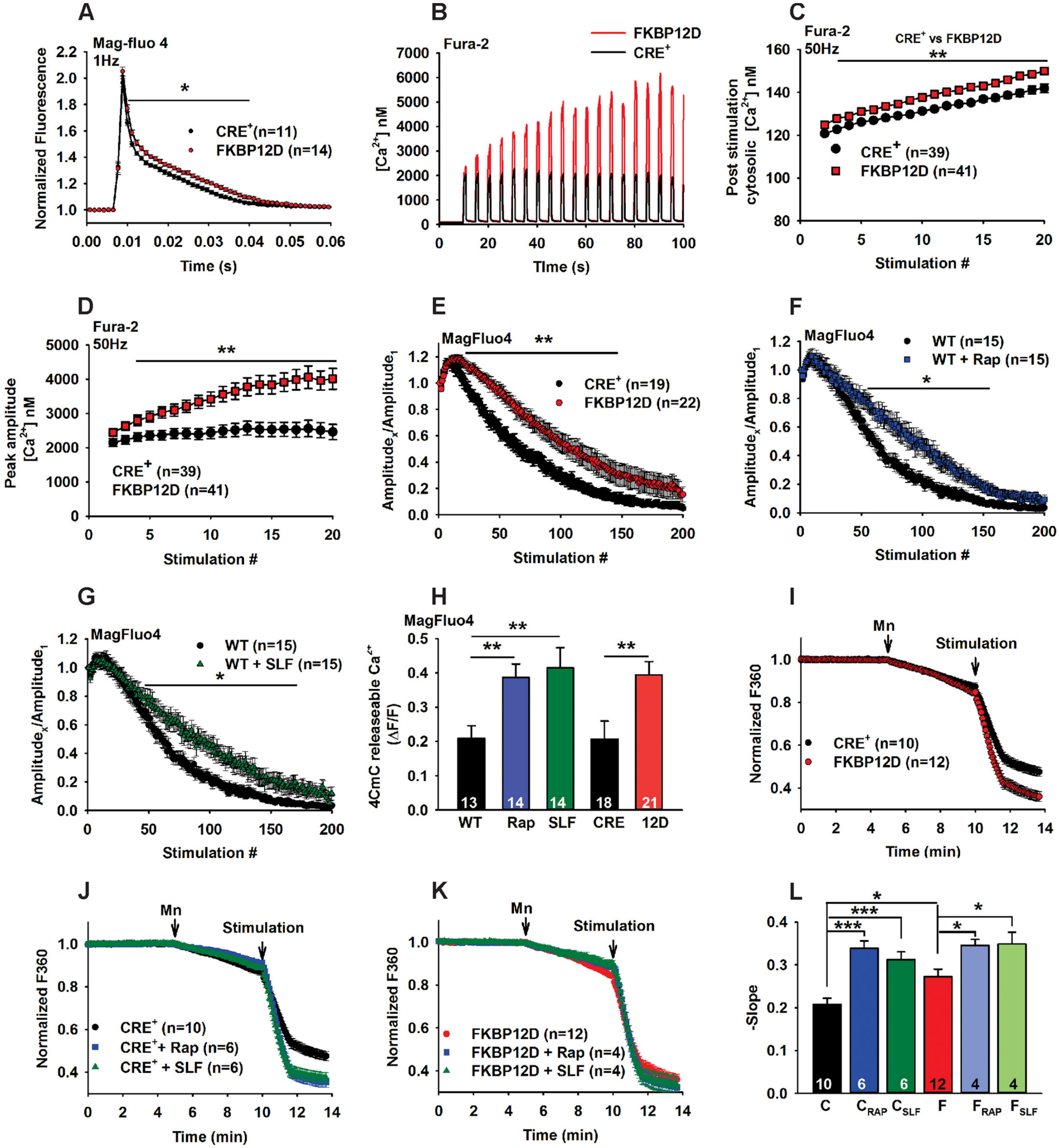

FIGURE 4. **Effect of FKBP12 deficiency, rapamycin and SLF on Ca^{2+} handling in FDB fibers.** *A*, effect of FKBP12 deficiency on the Ca^{2+} transient assessed with Mag-fluo4 fluorescence elicited by a single twitch stimulation (1 Hz). *B*, the effect of FKBP12 deficiency on the amplitude of the Ca^{2+} transients during repetitive 50-Hz fatiguing stimulation assessed with Fura-2. *C*, post-transient Ca^{2+} concentrations in fibers stimulated as described in *B*. *D*, analysis of the amplitude of the Ca^{2+} transients with repetitive stimulation. *E*, effects of fatiguing stimulation (100 Hz) on the amplitude of the Ca^{2+} transients in CRE^+ and FKBP12D fibers measured with Mag-fluo-4. *F*, effects of fatiguing stimulation on the amplitude of the Ca^{2+} transients in WT fibers treated with rapamycin and measured with Mag-fluo-4. *G*, effects of fatiguing stimulation on the amplitude of the Ca^{2+} transients in WT fibers treated with SLF and measured with Mag-fluo-4. Note that the *control curve* is the same as in *F* because SLF and rapamycin were each tested with each fiber preparation. *H*, 4-chloro-*m*-cresol (*4CmC*)-releasable Ca^{2+} measured immediately after the fatiguing stimulation of CRE^+ and FKBP12 or in WT fibers treated with low dose rapamycin or SLF. *I*, manganese quench and the effects of stimulation in fibers from CRE^+ and FKBP12D mice. *J*, manganese quench and the effects of stimulation in fibers treated with rapamycin or SLF from CRE^+ control mice. *K*, manganese quench and the effects of stimulation in fibers treated with rapamycin or SLF from FKBP12D mice. *L*, manganese quench slopes during stimulation and effects of rapamycin, SLF, and FKBP12 deficiency. *C*, CRE^+; *F*, FKBP12D. Data are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

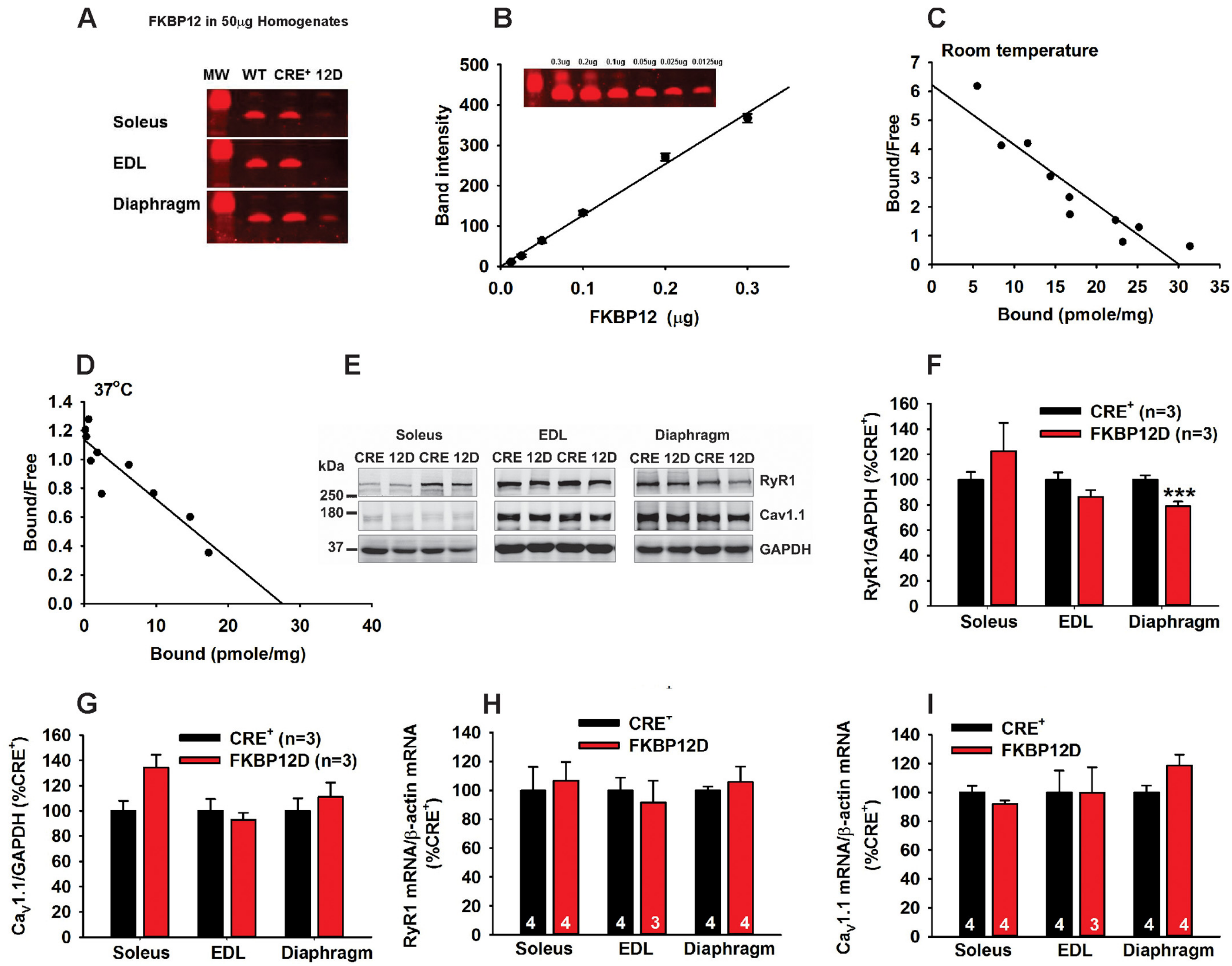

FIGURE 5. **Analysis FKBP12, RyR1, and $Ca_V1.1$ levels in FKBP12D and CRE+ mice.** *A*, FKBP12 in homogenates. *B*, FKBP12 standard curve. *C*, Scatchard analysis of [^{35}S]FKBP12 binding at room temperature to muscle membranes from WT mice. *D*, Scatchard analysis of [^{35}S]FKBP12 binding at 37 °C to muscle membranes from WT mice. *E*, Western blots for $Ca_V1.1$, RyR1, and GAPDH in muscles of 7-week-old FKBP12D and CRE+ mice. *F*, analysis of RyR1 protein normalized to GAPDH in soleus, EDL, and diaphragm of CRE+ and FKBP12D mice. *G*, analysis of $Ca_V1.1$ normalized to GAPDH in FKBP12D and CRE+ soleus, EDL, and diaphragm. *H*, RyR1 mRNA from quantitative RT-PCR. *I*, CaV1.1 mRNA from quantitative RT-PCR. Data are shown as mean ± S.E. (*error bars*).

Ser-236)/S6 and 4E-BP1 (Thr-37/Thr-46)/4E-BP1. We also found increases in phospho-Akt (Ser-474)/Akt, phospho-Akt (Thr-308)/Akt, phospho-ERK (Thr202/Tyr-204)/ERK/GAPDH, and L7a/GAPDH (a ribosomal protein marker). These findings demonstrate that FKBP12 deficiency, low dose rapamycin, and SLF all activate mTORC1, possibly upstream of Akt. The elevated phospho-Akt (Ser-473)/Akt and phospho-Akt (Thr-308)/Akt suggested that mTORC2 and the PI3K pathway activation led to downstream activation of mTORC1. However, the increased phosphorylation of ERK1/2 could have also contributed to the increases in phospho-S6 (Ser-235/Ser-236)/S6 and phospho-4E-BP1(Thr-37/Thr-46)/4E-BP1.

Although autophagy is also stimulated by mTORC1 activation, we did not find differences in beclin 1 or Atg5 levels in any muscle of the FKBP12D or rapamycin-treated mice (data not shown). We found a small increase in beclin 1 in the EDL muscle of SLF-treated mice but no change in Atg5 (data not shown). We conclude that autophagy is not activated by these treatments.

FKBP12 regulates TGFβR1 signaling, but other than a small increase in phospho-SMAD2 (Ser-465/Ser-467)/SMAD2 in the soleus after rapamycin treatment, we failed to detect any other changes in phospho-SMAD2 (Ser-465/Ser-467)/SMAD2 in muscles of FKBP12D mice or in mice treated with either rapamycin or SLF (data not shown), suggesting little change in TGFβ signaling.

Signal Transduction Pathways That Modulate Fiber Type Distribution—One of the major changes associated with the FKBP12D mice is an increase in type I fibers. We analyzed pathways known to regulate fiber type changes in the muscle (Fig. 9, *A–G*). We found no differences in the protein levels of ERRγ, a known contributor to the slow muscle program (34), in FKBP12D muscle compared with CRE+ muscle (Fig. 9*B*). However, muscle levels of ERRα, which has many overlapping functions with ERRγ, enhances PGC-1α signaling (35), and is involved in muscle repair (36), were elevated in the muscle of FKBP12D mice (Fig. 9*C*). PPARβ/δ also contributes to the slow fiber program (19) and is elevated in the muscle of FKBP12D mice (Fig. 9*D*). Both ERRα and

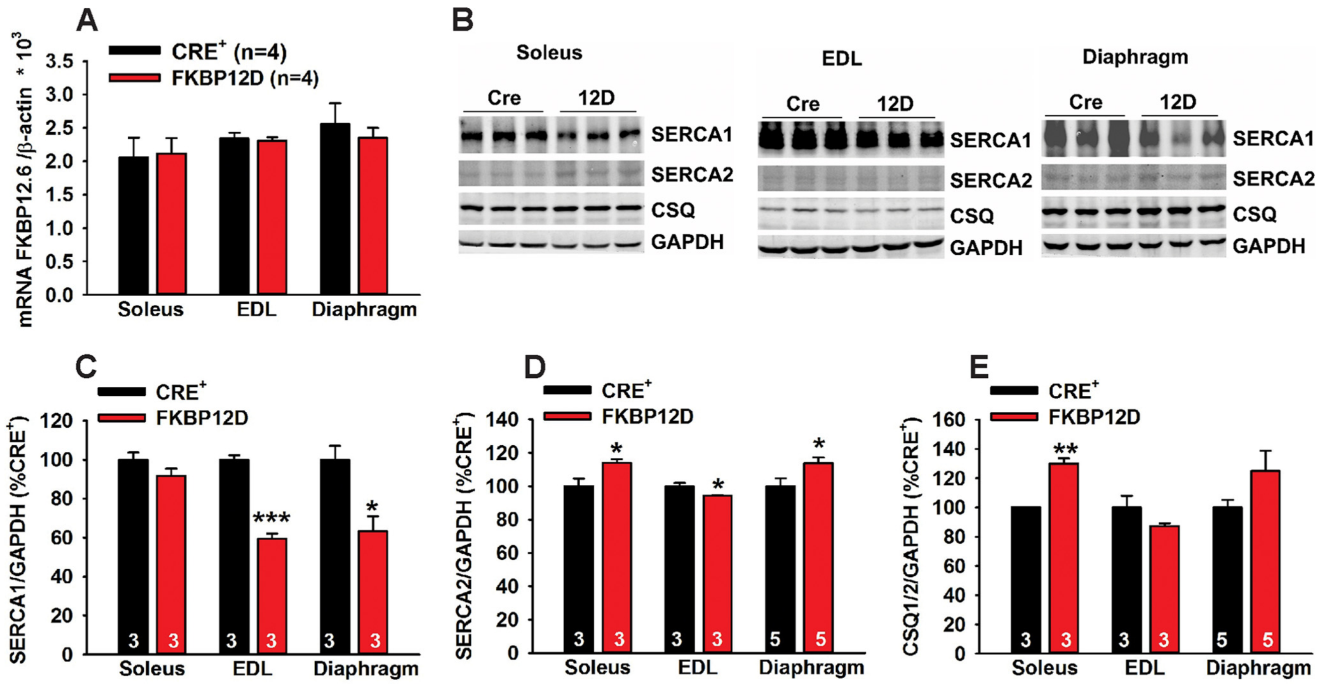

FIGURE 6. **Muscle levels of FKBP12.6, SERCA1, SERCA2, and calsequestrin.** *A*, mRNA for FKBP12.6 assessed by quantitative RT-PCR. FKBP12.6 protein levels are not detectable in skeletal muscle with the antibodies used. *B*, Western blots for SERCA1, SERCA2, and CSQ 1/2 (*CSQ*) in muscle of FKBP12D and CRE$^+$ mice. *C*, analysis of SERCA1 normalized to GAPDH and plotted as percentage of CRE$^+$. *D*, analysis of SERCA2 normalized to GAPDH and plotted as percentage of CRE$^+$. *E*, analysis of CSQ1/2 normalized to GAPDH and plotted as percentage of CRE$^+$. Data are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

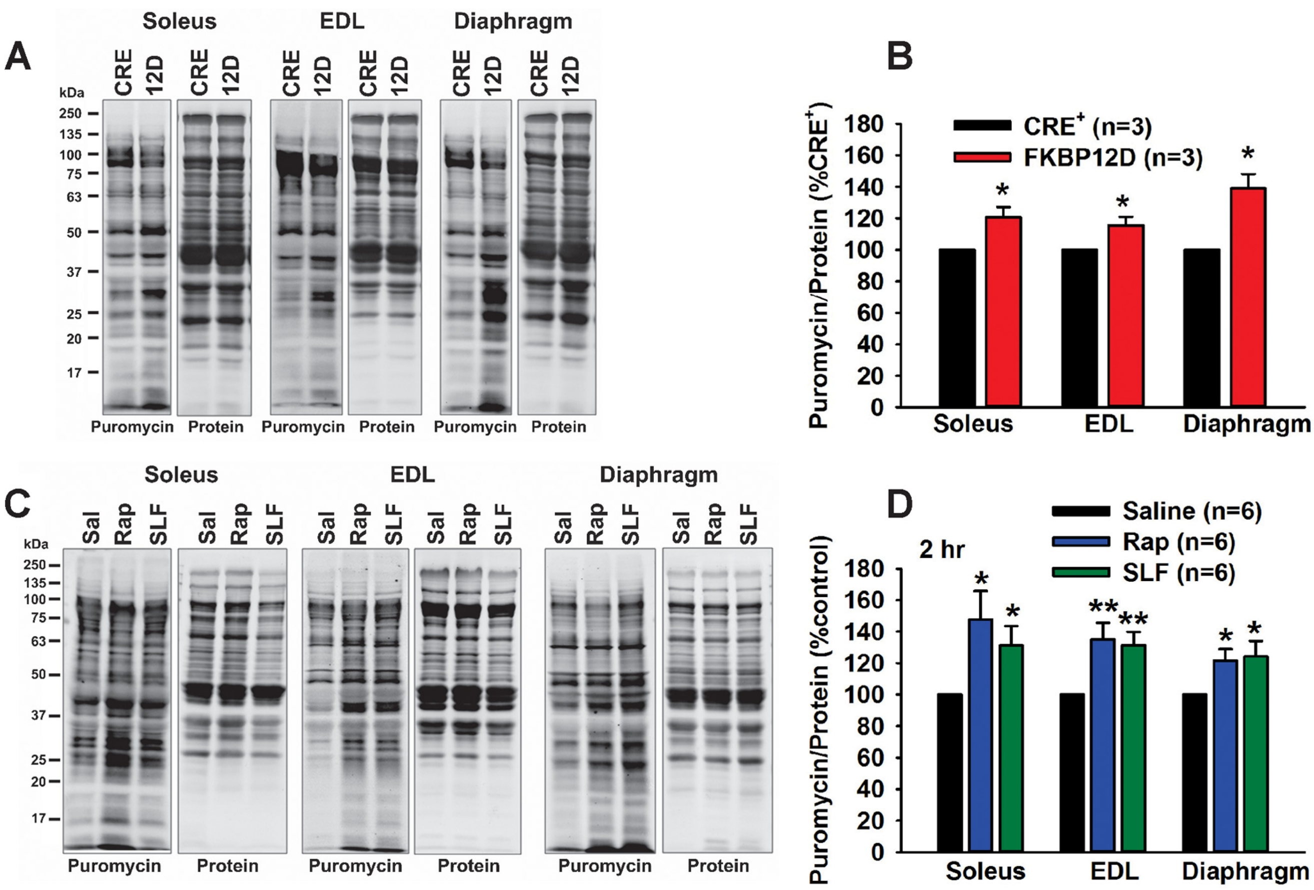

FIGURE 7. **Effects of FKBP12 deficiency and low dose rapamycin and SLF on protein synthesis.** *A*, Western blot for puromycin in muscle homogenates from CRE$^+$ and FKBP12D mice (6–8 weeks of age) after injection with puromycin as described under "Materials and Methods." Shown are both the Western blot for puromycin and the total protein stain. *B*, analysis of puromycin Western blots. *C*, Western blot for puromycin in mice treated with saline, rapamycin (10 μg/kg), or SLF (1 μg/kg). Mice (6–8 weeks of age) were injected with puromycin and sacrificed, muscle was isolated and homogenized, and the muscle lysates were used for Western blotting with anti-puromycin antibodies. *D*, analysis of puromycin Western blots. Data are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$.

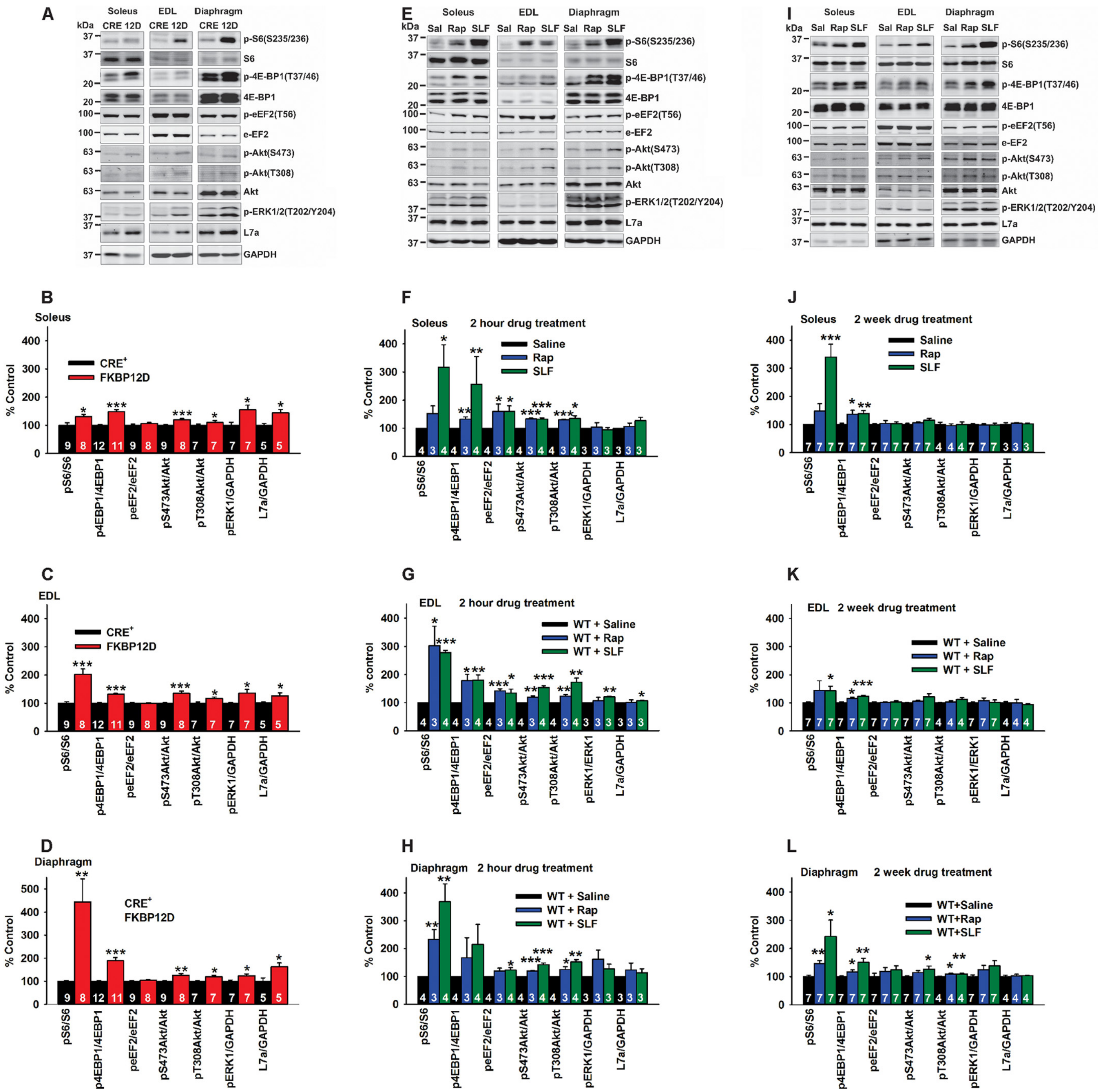

FIGURE 8. **Effects of FKBP12 deficiency and low dose rapamycin and SLF on signal transduction pathways that modulate protein synthesis.** *A*, representative Western blot of proteins that impact protein synthesis in FKBP12D and CRE$^+$ mice (8–10 weeks old). *B*, analysis of signal transduction changes in the solei of FKBP12D and CRE$^+$ mice. *C*, analysis of signal transduction changes in the EDLs of FKBP12D and CRE$^+$ mice. *D*, analysis of signal transduction changes in the diaphragms of FKBP12D and CRE$^+$ mice. *E*, representative Western blot for proteins that impact protein synthesis in muscle of mice treated with saline, rapamycin, or SLF for 2 h. *F*, analysis of signal transduction changes in the solei of saline-, rapamycin-, and SLF-treated mice. *G*, analysis of signal transduction changes in the EDLs of saline-, rapamycin-, and SLF-treated mice. *H*, analysis of signal transduction changes in the diaphragms of saline-, rapamycin-, and SLF-treated mice. *I*, representative Western blot for proteins that impact protein synthesis in muscle of mice treated with saline, rapamycin, or SLF for 2 weeks. *J*, analysis of signal transduction changes in the solei of mice treated with saline, rapamycin, or SLF for 2 weeks. *K*, analysis of signal transduction changes in the EDLs of mice treated with saline, rapamycin, or SLF for 2 weeks. *L*, analysis of signal transduction changes in the diaphragms of mice treated with saline, rapamycin, or SLF for 2 weeks. Data are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ERRγ mRNA levels were elevated in muscle of FKBP12D compared with CRE$^+$ mice (Fig. 9, *E* and *F*).

Calcineurin is thought to play a major role in fiber type changes in muscle, and we previously demonstrated an increase in calcineurin levels in FKBP12D mice (23). However, we were unable to detect differences in calcineurin activity between muscle homogenates of FKBP12D and CRE$^+$ mice (Fig. 9*G*). This does not preclude activation of calcineurin in muscle due to increases in Ca^{2+} because these changes cannot be assessed in the *in vitro* assay, where Ca^{2+} concentrations are controlled.

The fiber type changes that occurred after a 2-week treatment with SLF and rapamycin were small compared with those

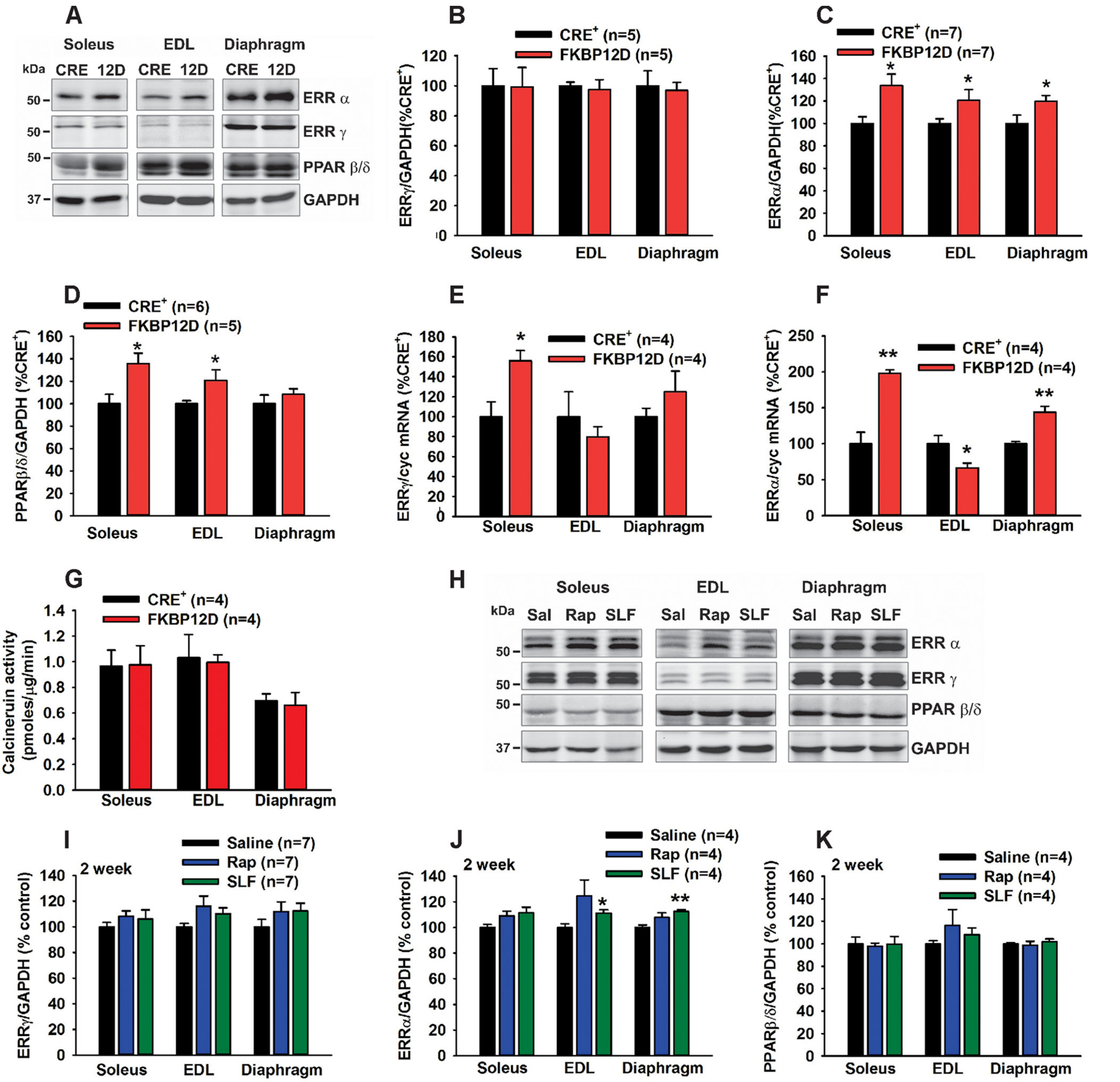

FIGURE 9. **Effect of FKBP12 deficiency, rapamycin, and SLF on pathways that regulate the slow fiber program.** *A*, Western blot for ERRα, ERRγ, PPARβ/δ, and GAPDH in muscles from FKBP12D and CRE$^+$ mice (8–10 weeks old). *B*, analysis of ERRγ levels. *C*, analysis of ERRα levels. *D*, analysis of PPARβ/δ levels. *E*, analysis of the effects of FKBP12 deficiency on the mRNA levels of ERRγ. *F*, analysis of the effects of FKBP12 deficiency on the mRNA levels of ERRα. *G*, calcineurin activity in muscles of CRE$^+$ and FKBP12D mice. *H*, Western blot for the effects of 2-week treatment with SLF and rapamycin on ERRα and ERRγ levels (8–10-week-old mice). *I*, analysis of ERRγ levels. *J*, analysis of ERRα levels. *K*, analysis of PPARβ/δ levels. Data are shown as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$.

seen in the muscle of FKBP12D mice. Not surprisingly, the changes in the proteins that drive the slow fiber program were also smaller (Fig. 9, *H–K*), and we only detected a significant difference in ERRα (Fig. 9*J*). No changes in these proteins were detected in mice treated for only 2 h with either rapamycin or SLF (data not shown).

DISCUSSION

In this paper, we explore the multiple roles of FKBP12 in skeletal muscle by examining the effects of FKBP12 deficiency and the drugs, rapamycin and SLF, that bind FKBP12. Most studies with rapamycin in mice use doses from 2 to 10 mg/kg body weight, doses often related to mTORC1 inhibition and decreased protein synthesis. In contrast, our data suggest that much lower concentrations of rapamycin (10 μg/kg) and the non-immunosuppressive FKBP ligand SLF (1 μg/kg) enhance refilling of SR Ca^{2+} stores to increase protein synthesis, slow muscle fatigue, and initiate up-regulation of type I fibers. A challenge when assessing the functional consequences of treatment with drugs such as rapamycin is to determine the specific

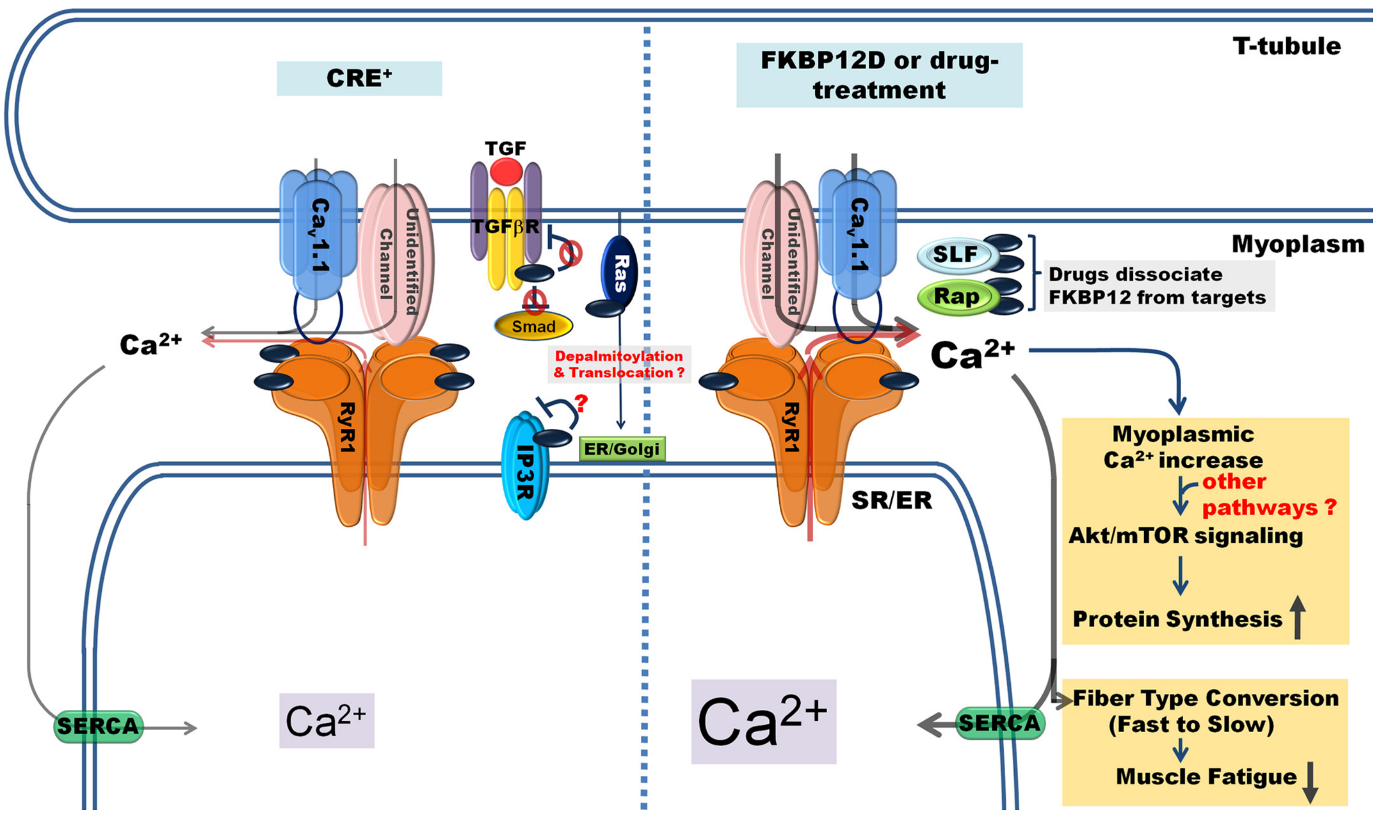

FIGURE 10. **A model for the effects of FKBP12 deficiency, low dose rapamycin, and SLF on muscle function.** We propose that depletion of FKBP12, low dose rapamycin, and SLF alter the conformation and/or Ca^{2+} leak via RyR1 and increase Ca^{2+} influx into the fiber. This added Ca^{2+} facilitates refilling of SR Ca^{2+} stores and prevents depletion during repetitive stimulation. Altered Ca^{2+} handling and/or other FKBP12 targets activate mTORC1 to increase protein synthesis and increases ERRα to activate the slow fiber program.

contributions of alterations in the multiple pathways regulated by FKBP12. We identify FKBP12-dependent changes in both myoplasmic Ca^{2+} handling and protein synthesis that are likely to impact multiple processes in the muscle fiber but found no changes in TGFβR1 signaling.

Both FKBP12 deficiency and low dose rapamycin and SLF improve skeletal muscle endurance, most likely due to the combined effects of enhanced refilling of SR Ca^{2+} stores (thereby slowing depletion with repetitive stimulation) and increased fatigue-resistant type I fibers, because both of these parameter are known to modulate muscle fatigue (18–21). Consistent with an increase in type I fibers, ERRα and PPARβ/δ are increased by FKBP12 deficiency and 2-week treatment with rapamycin and SLF. We detect small increases in the mRNA for ERRγ but no apparent changes in ERRγ protein levels. However, the possibility exists that changes in Ca^{2+} handling impact this signaling pathway because ERRγ has a predicted Ca^{2+} calmodulin binding site (see the Calmodulin Target Database Web site).

Low dose rapamycin (10 nM for a 1-h incubation with isolated muscle prior to the fatiguing protocol) also slows muscle fatigue in isolated diaphragm fibers, suggesting that, although the increase in type I fibers contributes to improved endurance, the fiber type changes are not required for slowing of muscle fatigue.

The major changes in Ca^{2+} handling in adult muscle fibers in response to low dose rapamycin, low dose SLF, or FKBP12 deficiency were increased Ca^{2+} influx, increased cytosolic Ca^{2+} concentrations, increased SR Ca^{2+} stores, and increased SR Ca^{2+} release. Previously, using myotubes, we found that FKBP12 deficiency increased voltage-gated Ca^{2+} influx but decreased SR Ca^{2+} release (23). The difference in SR Ca^{2+} release between myofibers and myotubes may reflect the lack of structural organization in myotubes (37) and/or differences in the expression levels of CRE+ recombinase and, hence, the efficiency of the knockdown of FKBP12 expression. Because our objective in this study was to assess the functional consequences of low dose rapamycin or SLF and FKBP12 deficiency for the function of adult skeletal muscle, all studies in this paper were performed with adult muscle fibers. Because the increased influx required electrical stimulation, the most likely candidate for mediating the Ca^{2+} influx is the voltage-dependent Ca^{2+} channel, $Ca_V1.1$. However, given the small magnitude of the Ca^{2+} current through $Ca_V1.1$ and its inactivation (38), it seems unlikely that $Ca_V1.1$ alone is responsible for the increased Ca^{2+} influx and enhanced Ca^{2+} store refilling, especially poststimulation (Fig. 4*A*). Additional channels that contribute to this influx remain to be identified. Regardless of whether the Ca^{2+} influx is due entirely to altered $Ca_V1.1$ or another Ca^{2+} channel, the increased Ca^{2+} influx to the muscle is likely to underlie the enhanced Ca^{2+} store refilling.

Bellinger *et al.* (31) found that FKBP12 association with RyR1 is reduced following a single bout of swimming exercise in mice. This effect was dose-dependent, increasing with increasing duration of exercise. In addition, with chronic (3 weeks) swim training, RyR1 *S*-nitrosylation increased, whereas FKBP12

association with RyR1 decreased. In this study, the effect of the swim training program on fatigue was measured using an entirely different mode of exercise (treadmill running), making assessment of the effects of the training program on endurance and fatigue difficult at best. When mice or humans are tested using the mode of exercise for which they have trained, adaptation typically occurs very rapidly. Thus, an alternative interpretation of the results of Bellinger *et al.* (31) is that RyR1 *S*-nitrosylation of RyR1 is part of the normal adaptation to endurance exercise training. The fatigue-slowing effects observed by Bellinger *et al.* (31) were also very small, and the drug used (S107) interacts with RyR1 to increase FKBP12 binding and slow passive SR Ca^{2+} leak. Our data suggest that low dose rapamycin (used clinically at much higher doses that we are using) and SLF interact with FKBP12 to alter the interaction of RyR1 with a Ca^{2+} influx channel. The effects of low dose rapamycin and SLF are both activity-dependent and transient and do not lead to sustained increases in cytosolic Ca^{2+}. The transient Ca^{2+} changes are beneficial rather than detrimental because Ca^{2+} influx enhances the refilling of SR Ca^{2+} stores to slow muscle fatigue. Although some of the slowing of fatigue in the diaphragm is due to fiber type changes (increased type I fibers), the ability of higher concentrations of rapamycin to increase the rate of fatigue in the FKBP12-deficient diaphragm suggests that there is also a more direct FKBP12-dependent effect.

It is likely that the transiently increased cytosolic Ca^{2+} concentrations associated with rapamycin/SLF treatments or FKBP12 deficiency contribute to the changes in protein synthesis because Ca^{2+} is required for mTORC1 activation of S6K (39–43). However, the mechanism for this Ca^{2+}-dependent regulation of mTORC1 remains to be elucidated. Rastogi *et al.* (44) found that rapamycin activated both Akt and ERK pathways in macrophages. Another possibility is that the drugs and FKBP12 deficiency increase the activity of Ras at the membrane by promoting its membrane retention and, through the increase in cytosolic Ca^{2+}, activate guanosine nucleotide exchange factor, RasGRP (Ras guanyl-releasing protein 1). RasGRP is regulated by both Ca^{2+} and diacylglycerol (45). Activated Ras could, in turn, activate ERK signaling. The increased ERK signaling by FKBP12 and low dose rapamycin and SLF supports a role for this pathway, and studies are currently under way to determine whether treatment of skeletal muscle fibers with low dose rapamycin or SLF affects Ras localization and phospholipase C activity. Consistent with this pathway, treatment of PC12 cells with FK506 enhances Ras signaling in PC12 cells (46).

In summary (Fig. 10), our findings suggest that beneficial effects of decreased muscle FKBP12 and low dose rapamycin and SLF on adult skeletal muscle function are, at least partially, due to altered regulation of myoplasmic Ca^{2+} handling and increased protein synthesis. Other FKBP12-binding proteins may also contribute to the phenotype. However, we find no evidence of alteration in TGFβ signaling. The current study advances our understanding of skeletal muscle fatigue and regulation of muscle protein synthesis and lays the groundwork for the development of therapeutic interventions for patients with symptoms characterized by enhanced fatigue.

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FEDERAL FINANCIAL REPORT

(Follow form instructions)

1. Federal Agency and Organizational Element to Which Report is Submitted USA Medical Research Acquisition Activity	2. Federal Grant or Other Identifying Number Assigned by Federal Agency (To report multiple grants, use FFR Attachment) DAMD W81XWH 10-2-0117	Page 1	of 1 pages

3. Recipient Organization (Name and complete address including Zip code)

Baylor College of Medicine One Baylor Plaza, Houston, Texas 77030

4a. DUNS Number	4b. EIN	5. Recipient Account Number or Identifying Number (To report multiple grants, use FFR Attachment)	6. Report Type	7. Basis of Accounting
'051113330	1741613878A1	1330003333	Quarterly ☐ Semi-Annual ☐ Annual X Final	X Cash ☐ Accrual

8. Project/Grant Period From: (Month, Day, Year)	To: (Month, Day, Year)	9. Reporting Period End Date (Month, Day, Year)
10/01/10	9/30/2014	9/30/2014

10. **Transactions**	Cumulative
(Use lines a-c for single or multiple grant reporting)	
Federal Cash (To report multiple grants, also use FFR Attachment):	
a. Cash Receipts	$1,281,348.00
b. Cash Disbursements	$1,281,348.00
c. Cash on Hand (line a minus b)	$0.00
(Use lines d-o for single grant reporting)	
Federal Expenditures and Unobligated Balance:	
d. Total Federal funds authorized	$1,281,348.00
e. Federal share of expenditures	$1,281,348.00
f. Federal share of unliquidated obligations	
g. Total Federal share (sum of lines e and f)	$1,281,348.00
h. Unobligated balance of Federal funds (line d minus g)	$0.00
Recipient Share:	
i. Total recipient share required	$0.00
j. Recipient share of expenditures	$0.00
k. Remaining recipient share to be provided (line i minus j)	$0.00
Program Income:	
l. Total Federal program income earned	$0.00
m. Program income expended in accordance with the deduction alternative	$0.00
n. Program income expended in accordance with the addition alternative	$0.00
o. Unexpended program income (line l minus line m or line n)	$0.00

11. Indirect Expense	a. Type	b. Rate	c. Period From	Period To	d. Base	e. Amount Charged	f. Federal Share
	Predetermined	53.5	10/1/2010	9/30/2014	536,065.98	300,170.30	300,170.30
				g. Totals:	536,065.98	300,170.30	300,170.30

12. *Remarks: Attach any explanations deemed necessary or information required by Federal sponsoring agency in compliance with governing legislation:*

13. Certification: By signing this report, I certify that it is true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent information may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 218, Section 1001)

a. Typed or Printed Name and Title of Authorized Certifying Official 12/21/2014, 2:15 PM Chryll Batiste Chryl Batiste , Accounting Manager, Grants and Contracts, BCM	c. Telephone (Area code, number and extension) 713-798-4369 d. Email address cbatiste@bcm.edu
b. Signature of Authorized Certifying Official	e. Date Report Submitted (Month, Day, Year)
	14. Agency use only:

Standard Form 425
OMB Approval Number: 0348-0061
Expiration Date: 10/31/2011